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**The Vaccinia Virus I5L Protein is Targeted to Viral Factories During Infection
and Anchored into Membranes**

by

Scott Fraser Slemko



A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of
the

requirements for the degree of *Master of Science*

in

Virology

Department of Medical Microbiology and Immunology

Edmonton, Alberta

Fall 2003



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Sept. 20, 2003.

Abstract

Vaccinia virus, the prototypical poxvirus, has a complex morphogenetic pathway that is poorly defined. Unresolved questions regarding the vaccinia virus lifecycle such as entry into host cells, virion assembly, and virion egress are intimately connected to the uncertainties surrounding the assembly pathway and the resulting structure of nascent virions. We have studied the intracellular targeting of the vaccinia virus I5L protein which we believe is involved in virion morphogenesis. We have shown that this protein targets to cytoplasmic sites of virion assembly during infection in a process mediated by the hydrophobic N-terminus, while the hydrophobic C-terminus anchors I5L to membranes both *in vitro* and in cell culture.

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List of Abbreviations

araC	cytosine arabinoside
ATP	adenosine triphosphate
Bcl-2	B-cell lymphoma protein 2
BGMK	baby green monkey kidney
BHK	baby hamster kidney
Cb5	cytochrome b5
CEV	cell-associated enveloped virion
CPMMs	canine pancreatic microsomal membranes
CTL	cytotoxic T-lymphocyte
DMEM	Dulbecco's Modified Eagle Medium
dsDNA	double-stranded deoxyribonucleic acid
EEV	extracellular enveloped virion
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
EGFR	epidermal growth factor receptor
ER	endoplasmic reticulum
ERGIC	endoplasmic reticulum-Golgi intermediate compartment
EV	ectromelia virus
FBS	fetal bovine serum
GFP	green fluorescent protein
gpt	guanine phosphoribosyltransferase
GTP	guanosine triphosphate
HCMV	human cytomegalovirus
HLA-E	human lymphocyte antigen-E

IFN	interferon
IgG	immunoglobulin G
IMV	intracellular mature virion
IV	immature virion
KCS	Kozak's consensus sequence
LMP	low melting point
LSDV	lumpy skin disease virus
MCV	molluscum contagiosum virus
MHC-I	major histocompatibility complex-I
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
NCBI	National Center for Biotechnology Information
NCS	newborn calf serum
NF-kB	nuclear factor kappa-B
NK	natural killer
NP-40	Nonionic detergent P-40
ORF	open reading frame
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
RFV	rabbit fibroma virus
RNase	ribonuclease
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SRP	signal recognition particle
SRPR	signal recognition particle receptor
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor

TAP	transporter for antigen presentation
TNT	Transcription and Translation
UTR	untranslated region
UV	ultraviolet
Vamp	vesicle-associated membrane protein
VV	vaccinia virus
YLDV	yaba-like disease virus

1. Introduction

Throughout history, poxviruses have remained an important virus to humankind. It is estimated that smallpox, the most notorious member of the *Poxviridae* family, is responsible for more human deaths in recorded history than any other infectious disease (Fenner, 1998). The eradication of smallpox from the human population was achieved in 1978, but the threat of accidental or intentional reintroduction of this virus persists (Lane *et al.*, 2001).

Aside from the potential human health threat that poxviruses pose, much of the interest in poxviral research stems from the fact that poxviruses employ a vast array of interesting and effective immune evasion strategies (Smith *et al.*, 1997). Poxviruses thus serve as model organisms for studying virus-host interactions.

Although poxviruses cause devastating infections in a wide range of species, they have recently emerged as promising therapeutic tools (Paoletti, 1996; Broder and Earl, 1999; Stittelaar and Osterhaus, 2001). The ease with which recombinant poxviruses are generated and their ability to express foreign genes *in vivo* has made them attractive viruses for medical researchers (Broder and Earl, 1999). Vaccinia virus and canarypox virus have been widely used as gene-therapy vectors and have also been used in the development of vaccines against a wide range of pathogens (Paoletti, 1996; Liljeqvist and Stahl, 1999; Cohen, 2003). In addition, a vaccinia virus mutant is being developed as an oncolytic agent, able to selectively replicate in and destroy cancerous tissue (Zeh and Bartlett, 2002).

Efforts to improve and expand poxvirus-based therapies are often hampered by our incomplete understanding of poxvirus biology. Although poxvirus research has been active for nearly 50 years, certain aspects of the poxvirus life cycle are still poorly understood, particularly virus entry, assembly and immune evasion. Research into these

areas will allow researchers to develop more effective therapeutic tools and learn more about virus assembly and virus-host interactions in general.

1.1 The *Poxviridae*

The *Poxviridae* represent a diverse family of viruses with the ability to infect a wide range of host species. This family is divided into two subfamilies, the *Chordopoxvirinae* and *Entomopoxvirinae*, which infect vertebrates and invertebrates, respectively. The *Chordopoxvirinae* are further divided into 8 genera: *Orthopoxviridae*, *Leporipoxviridae*, *Parapoxviridae*, *Capripoxviridae*, *Molluscipoxviridae*, *Suipoxviridae*, *Yatapoxviridae*, and *Avipoxviridae* (Table 1). Most of what is known about poxvirus biology has been gleaned by studying the orthopoxvirus and leporipoxvirus genera.

1.2 The Orthopoxviruses

Variola virus, a member of the orthopoxvirus genus, is the most notorious member of the poxvirus family and is the causative agent of smallpox. Variola virus strains are divided into two groups: the variola major strains which exhibit 20-30% morbidity, and the variola minor strains, which exhibit 1-2% morbidity (Fenner, 1996). Variola major's high morbidity, coupled with its high transmissibility via aerosolized droplets, has made it a significant threat to human health in the past (Fenner, 1996). The World Health Assembly sponsored a global eradication effort in 1958 with a mandate to eradicate smallpox from the human population using vaccinia virus to vaccinate against smallpox (Fenner *et al.*, 1998). In 1979, after a long struggle, the world was declared officially free of endemic smallpox (Wehrle, 1980).

Variola virus is not the only public health threat in the poxvirus family. Monkeypox, another member of the *Orthopoxviridae*, endemic to Western and Central Africa, is able to infect humans and cause a disease reminiscent of smallpox (Fenner,

Subfamily	Genus	Member viruses
* <i>Chordopoxvirinae</i>	<i>Orthopoxviridae</i>	Vaccinia*, variola*, monkeypox*, cowpox*, camelpox, ectromelia, racoonpox, skunkpox, taterapox, Uasin Gishu, volepox
	<i>Leporipoxviridae</i>	Myxoma, rabbit fibroma
	<i>Parapoxviridae</i>	Auzduk disease, chamois contagious ecthyma, orf, pseudocowpox, parapox of deer, sealpox
	<i>Capripoxviridae</i>	Sheeppox, lumpy skin disease, goatpox
	<i>Molluscipoxviridae</i>	Molluscum contagiosum*
	<i>Yatapoxviridae</i>	Yaba-like disease*, tanapox, yaba monkey tumor
	<i>Suipoxviridae</i>	Swinepox
<i>Entomopoxvirinae</i>	<i>Avipoxviridae</i>	Canarypox, fowlpox, juncopox, mynahpox, pigeonpox, psittacinpox, quailpox, peacockpox, penguinpox, sparrowpox, starlingpox, turkeypox
	<i>Entomopoxviridae A</i>	Melolontha melolontha†
	<i>Entomopoxviridae B</i>	Amsacta moorei†
	<i>Entomopoxviridae C</i>	Chironimus luridus†

Table 1. The *Poxviridae* family. (*) Denotes those viral species reported to cause disease in humans. (†) Prototypal member. (Adapted from Moss B., 1996. *Poxviridae: The Viruses and Their Replication* in Field's Virology 3rd Ed.).

1996; Heymann *et al.*, 1998). Mortality rates of 2-10% are much lower than the 20-30% seen with smallpox, and although person-to-person transmission of monkeypox is relatively inefficient (Pattyn, 2000), the incidence of monkeypox cases is slowly, but steadily increasing, raising concerns that this virus may someday become a significant health threat (Meyer *et al.*, 2002). Camelpox virus is another member of the orthopoxvirus genus and is extremely lethal in camels, but has not been reported to infect humans (Gubser and Smith, 2002). Interestingly, phylogenetic analysis of the camelpox genome has indicated that this virus is the closest known relative of variola virus (Gubser and Smith, 2002). A comparison of genome structure has indicated that although camelpox and variola share all open reading frames, 11 of these are fragmented and likely non-functional in variola (Gubser and Smith, 2002).

1.2.1 Vaccinia

Vaccinia virus, the prototypical orthopoxvirus, is the best-characterized member of the *Poxviridae* family (Moss, 1996). It replaced cowpox as the virus of choice for immunizing people against smallpox (Fenner, 1996). In humans, vaccinia virus causes harmless, isolated pustules, followed by clearance of the virus by the host, providing immunity against other orthopoxviruses (Fenner, 1996). On rare occasions, more serious, and sometimes fatal systemic infections result, usually related to immunodeficiencies in the infected individual (Fenner, 1996). Although vaccinia is able to infect many different mammalian species, the natural host remains unknown. Complete genome sequencing of vaccinia confirms its classification as an orthopoxvirus, and also indicates that vaccinia virus is a distinct species in the family tree (Goebel *et al.*, 1990; Antoine *et al.*, 1998). The ease with which vaccinia is grown in the laboratory, its safety, and its widespread use in smallpox vaccination, have made it an attractive model virus for studying poxvirus biology.

1.2.2 Myxoma and Ectromelia Viruses

Myxoma virus is the prototypical member of the *Leporipoxviridae*. It causes a harmless infection in South American rabbits (*Sylvilagus* spp.), but an almost uniformly fatal disease, called myxomatosis, in European rabbits (*Oryctolagus cuniculus*) (Hayes and Richardson, 2001). Myxoma virus infection of European rabbits has been used to study virus-host interactions, particularly those involving immune evasion strategies of the virus (Nash *et al.*, 1999). This has helped to overcome the limitations of studying virus-host interactions with vaccinia for which the natural host is unavailable, or with variola, for which humans are the only readily infectible host. Since many of the immune evasion genes discovered in myxoma are found in other poxviruses, these studies have been extrapolated to other poxviruses, and in some cases to distantly related virus families.

In addition to myxoma virus, ectromelia virus provides researchers with another physiologically relevant animal model (Fenner and Buller, 1997). Ectromelia virus is a member of the orthopoxvirus genus, and causes a highly lethal infection in mice and other rodents. Since ectromelia shares a large number of immune evasion gene candidates with other members of the poxvirus family, notably vaccinia and variola, it is a useful animal model for studying virus-host interactions (Fenner and Buller, 1997).

1.3 The Poxvirus Genome

Mammalian poxvirus genomes (Figure 1) are large by viral standards and in the range of 150-250 kbp (Moss, 1996). They encode roughly one gene per kilobase, making the poxvirus proteome very complex. The genomes consist of linear dsDNA containing terminal inverted repeats. The central region of the genome is rich in essential proteins which include proteins involved in viral transcription, genome replication and virion

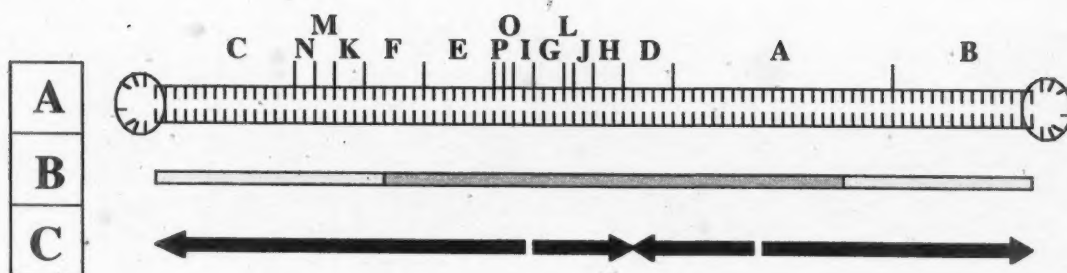


Figure 1. Hind III restriction endonuclease map of vaccinia virus. A) The standard convention for vaccinia gene nomenclature is derived from the Hind III restriction map of vaccinia strain Copenhagen. The largest restriction fragment is named fragment A, the second largest is named B and so on, down to fragment P. The ORFs within each fragment are numbered from left to right. By convention, genes read towards the right of the genome are given an R designation, while those read towards the left are given an L designation. B) Regions enriched in essential proteins are indicated in red, while regions enriched in host-range factors and immune evasion genes are indicated in green. C) With few exceptions, the orientation of genes are grouped into distinct regions as indicated by the arrows. The importance of this arrangement is unclear, but conservation is seen throughout the *Poxviridae*.

formation (Goebel *et al.*, 1990). It is therefore not surprising that this region is very highly conserved amongst the *Poxviridae*. The regions flanking the genome core are more variable between species and include many of the immune evasion genes and host-range factors (Perkus *et al.*, 1991; Massung *et al.*, 1993).

1.4 The Poxvirus Virion

The virion particles of poxviruses are the largest of any described animal virus (Figure 2). They appear brick-shaped with rough, studded surfaces, and measure 350 by 270 nm under cryoelectron microscopy and are estimated to possess over 100 different polypeptides (Roos *et al.*, 1996). The virion core is an amorphous mixture of viral genomic DNA and proteinaceous material. The core is dumbbell-shaped with protein-rich "lateral bodies" filling the concavities of the dumbbell and is surrounded by a phospholipid bilayer of controversial origin. Acquisition of this bilayer results in intracellular mature virion (IMV) formation (Dales and Mosbach, 1968; Sarov and Joklik, 1973). Some virions also acquire additional bilayers from the trans-Golgi compartment, resulting in formation of extracellular enveloped virions (EEV) (Schmelz *et al.*, 1994; Smith *et al.*, 2002).

1.5 Vaccinia Virus Lifecycle

The vaccinia lifecycle is complex, and requires a vast array of viral proteins. The various stages of the vaccinia lifecycle include attachment to a host cell, entry, gene transcription, DNA replication, virus assembly, and finally egress and dissemination (Figure 3). Throughout all of these stages, vaccinia must successfully evade or disarm the multitude of host immune defences.

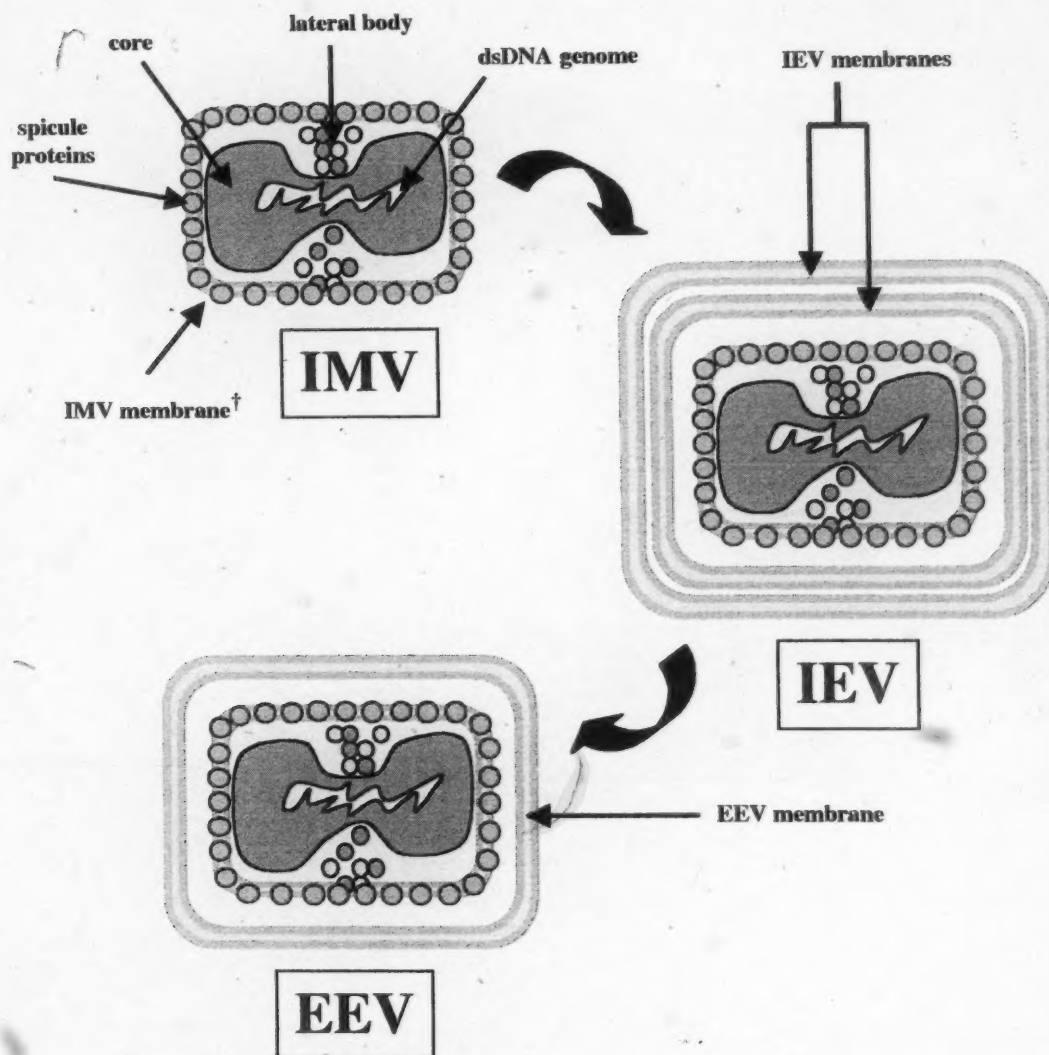


Figure 2. Structure of the different infectious forms of vaccinia virus

Intracellular mature virions (IMV) are fully infectious upon cell lysis. A small proportion of IMV are further wrapped with two additional bilayers derived from the *trans*-Golgi compartment to form intracellular enveloped virions (IEV). IEV lose one of these additional bilayers upon fusion with the inside of the plasma membrane, thus releasing an extracellular enveloped virion (EEV), which mediates long-range dissemination of the virus.

† There is disagreement in the poxvirus field as to whether the IMV membrane is a single bilayer, or is comprised of two tightly apposed bilayers.

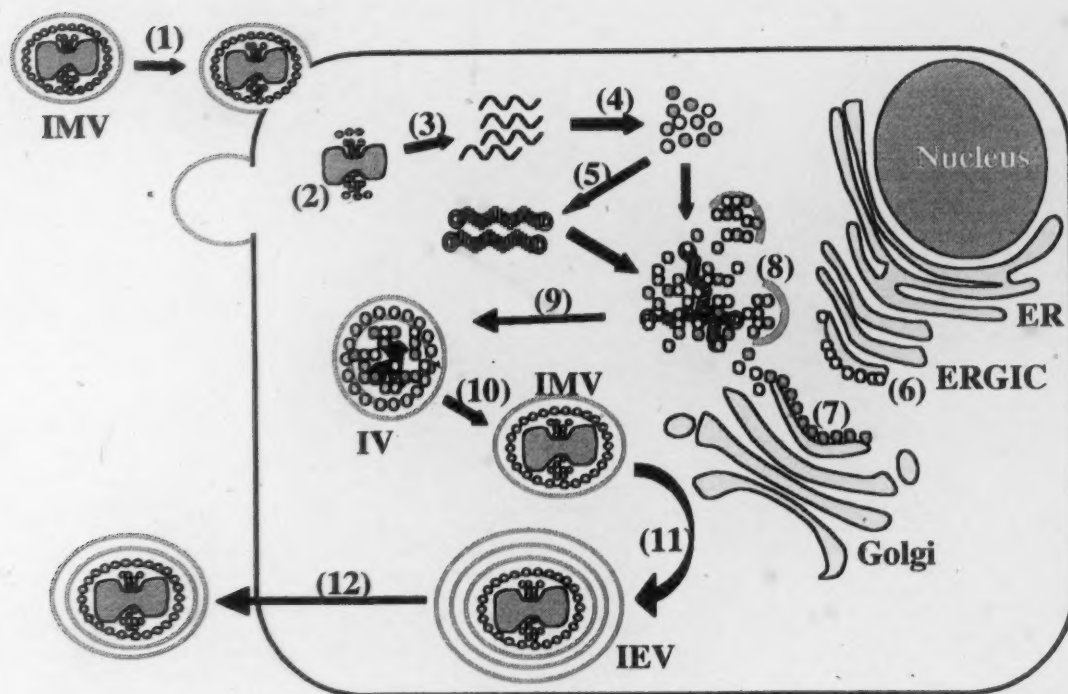


Figure 3. The vaccinia virus life cycle. Ligands on the outer surface of the virion bind to the target cell to initiate viral entry (1). Viral membranes are shed at the cell surface, releasing naked cores into the cell (2). Viral transcription and translation occur within the cytoplasm. Viral polymerases transcribe viral genes (3), which are translated to yield both housekeeping proteins and virion components (4). Some of these proteins replicate the viral genome (5), while others accumulate in the ERGIC (6), Golgi (7), or viral factories (8) where they initiate viral assembly or are destined to be incorporated into the virion as enzymatic components. Membranous viral crescents appear in viral factories (8), which wrap protein-rich viroplasmic material, as well as viral genomic material, to yield an immature virion (IV) (9). Several proteolytic cleavages and phosphorylation events take place within the IV to yield an intracellular mature virion (IMV) (10). Although IMVs are fully infectious particles upon cell lysis, they can be further wrapped with two additional bilayers derived from the trans-Golgi compartment to yield intracellular enveloped virions (IEV) (11). IEV fuse with the plasma membrane, resulting in loss of the outermost bilayer and release of an extracellular enveloped virion (EEV) (12). Some EEV remain attached to the cell surface and are called cell-associated enveloped virions (CEV).

1.5.1 Virus Entry

Vaccinia virus entry into cells is still a poorly understood process which has been complicated by the existence of two infectious forms of virus, IMV and EEV. IMV particles possess a single phospholipid shell surrounding the core (Dales and Mosbach 1968, Hollinshead *et al.*, 1999), while EEV possess an additional phospholipid bilayer surrounding the core (Schmelz *et al.*, 1994; Smith *et al.*, 2002). The simplest model for IMV entry involves fusion of the IMV with the plasma membrane resulting in release of the core into the cytoplasm (Doms *et al.*, 1990) (Figure 3). Although it is generally accepted that naked cores are released into cells, leaving the outer membranes at the surface, several recent studies have found little evidence to support membrane fusion (Pedersen *et al.*, 2000; Locker *et al.*, 2000). Electron microscopy (EM) studies have shown intact bundles of viral membranes at the cell surface and an absence of viral protein markers embedded in the plasma membrane (Pedersen *et al.*, 2000, Locker *et al.*, 2000).

EEV are surrounded by an additional phospholipid bilayer (Payne and Norrby, 1978; Schmelz *et al.*, 1994). A simple membrane fusion model for EEV predicts that fusion with a host cell would result in loss of this additional bilayer and release of, essentially, a fully wrapped IMV particle into the cell. This has not been observed to occur (Payne and Norrby, 1978; Doms *et al.*, 1990). Instead, naked cores are released into the cell upon EEV binding, just as with IMV entry, leaving both viral membranes at the cell surface, suggesting that IMV and EEV particles enter the cell using different mechanisms (Locker *et al.*, 2000).

The search for a cellular receptor for viral docking has thus far proven unsuccessful. A strong candidate is the epidermal growth factor receptor (EGFR) (Eppstein *et al.*, 1985; Marsh *et al.*, 1987). Competitive inhibition studies have shown

that exogenously added EGF inhibits viral plaque formation, however inhibition is only partial (Buller *et al.*, 1988). Furthermore, cells lacking the EGFR are still infectible by wild-type vaccinia virus suggesting that other cellular receptors must also play a role (Buller *et al.*, 1988).

The viral protein(s) responsible for virus attachment to cells have not been completely delineated. The viral D8L outer membrane protein mediates viral attachment to a wide variety of cells (Gong *et al.*, 1990; Maa *et al.*, 1990), but a D8L antibody or soluble D8L are not able to fully block viral plaque formation (Hsiao *et al.*, 1999), suggesting other viral surface ligands must be involved. Antibody neutralization studies have also implicated several other virion membrane proteins in viral entry. These include L1R (Ichihashi and Oie, 1996), A17L (Wallengren *et al.*, 2001) and A27L (Chung *et al.*, 1998). Their specific role(s) in viral entry are unknown, but their blockage with neutralizing antibodies only inhibits IMV, not EEV infection. Likewise, neutralizing antibodies to EEV specific proteins, A33R, A34R, A36R, A56R, B5R, F12L, and F13L only inhibited infection with EEV, not IMV (Law and Smith, 2002). These results suggest that more than one viral protein is involved in viral attachment to host cells, and that IMV and EEV use different mechanisms for attachment and entry.

1.5.2 Uncoating and Early Gene Expression

Once naked virion cores are released into the cytosol, they begin to synthesize early viral mRNA (Figure 3). Early transcripts and viral genomic material pass through breaches in the core wall into the cytoplasm (Moss, 1996). Transcription of vaccinia genes occurs entirely in the cytoplasm in the absence of cellular transcription machinery. The virus therefore supplies its own transcription machinery which is included in the viral particle as non-structural virion components (Munyon *et al.*, 1967) (Table 2). This machinery includes everything the virus needs for mRNA production including

<u>Virion Component</u>	<u>Function</u>
A5R	RNA polymerase subunit
A7L	early transcription factor
A18R	DNA helicase
A24R	RNA polymerase subunit
A29L	RNA polymerase subunit
B1R	protein kinase
D1R	capping enzyme
D6R	early transcription factor
D7R	RNA polymerase subunit
D11L	DNA helicase
D12L	capping enzyme
E1L	poly(A) polymerase
E4L	RNA polymerase subunit
F10L	protein kinase
G1L	metalloproteinase
G5.5R	RNA polymerase subunit
H1L	serine/tyrosine phosphatase
H4L	RNA polymerase subunit
H6R	DNA topoisomerase I
I7L	proteinase
I8R	DNA/RNA helicase
J3R	poly(A) polymerase, RNA methyltransferase
J4R	RNA polymerase subunit
J6R	RNA polymerase subunit
O2L	glutaredoxin
?	nicking-joining enzyme

Table 2. Enzymatic virion components. Reported enzymatic vaccinia virion components are listed along with their demonstrated or putative function.

transcription factors, polymerases, helicases, capping enzymes and polyadenylation factors.

Early mRNAs are detectable approximately 20 minutes after initial infection and represent the expression of about half of the poxvirus genome (Baldick and Moss, 1993). Genes expressed during this period encode many of the viral proteins necessary for host protein synthesis shut-off and to counter host anti-viral responses (Moss, 1996). Other early proteins include those responsible for viral DNA replication and intermediate gene expression (Evans and Traktman, 1987; McDonald *et al.*, 1992; Sanz and Moss, 1999).

1.5.3 Intermediate Gene Expression

At approximately 100 minutes post-infection transcription of intermediate mRNAs occurs (Vos and Stunnenberg, 1988). These represent a small set of genes mainly involved in the initiation of late gene expression (Passarelli *et al.*, 1996). They include several transcription factors, a helicase and a dsDNA binding protein (Davis and Mathews, 1993). It is during this time that viral DNA replication begins. This ultimately leads to the production of approximately 10,000 genome copies per infected cell, half of which eventually become wrapped into virions (Moss, 1996).

1.5.4 Late Gene Expression

Late gene expression begins at about 140 minutes post-infection and is regulated by viral DNA replication (Keck *et al.*, 1990). Unsuccessful viral DNA replication, as in the presence of the DNA synthesis inhibitor cytosine arabinose (araC), results in a failure to initiate late gene transcription (Taddie and Traktman, 1993). Unlike early and intermediate gene expression, late gene expression is not transient, but is rather sustained at high levels for the remainder of the infection (Moss, 1996). Late genes encode virion components, both structural and non-structural. Structural components include those

which form the virion core, core wall, and envelope (Table 3). Non-structural components include virion enzymes such as proteinases, kinases and early-gene transcription machinery (Table 2). Once late proteins are translated, virion assembly can begin.

1.5.5 Vaccinia Morphogenesis I – Crescent formation

Viral DNA replication occurs exclusively in the cytoplasm in a region adjacent to the trans-Golgi compartment in infected cells (Figure 3). This area has been termed the viral factory and becomes rich in viral DNA and electron-dense viroplasm as visualized by electron microscopy (Gaylord and Melnick, 1953; Joklik and Becker, 1964). As this viroplasm expands, crescent-shaped viral membranes are assembled through a poorly understood process (Dales and Mosbach, 1968). Crescents are covered by proteinaceous spicules on the convex surface and begin to accumulate dense viroplasm in the concavity (De Harven and Yohn, 1966). The crescent-shaped membrane grows and eventually encloses the DNA-rich viroplasm to form a spherical immature virion (IV) (Dales and Mosbach, 1968; Tsuruhara, 1971).

The mechanism of crescent formation has been a contentious issue in the poxvirus community. The debate centers around two interrelated issues: the source of the membranes for crescent formation, and whether crescents are composed of a single bilayer or two tightly apposed bilayers. Under electron microscopy, the crescents are not connected to any cellular membranous organelles (Dales and Mosbach 1968; Hollinshead *et al.*, 1999). This has led to the hypothesis that crescent membranes are synthesized *de novo* from phospholipid precursors, a theory without precedent in biology (Dales and Mosbach 1968; Stern and Dales, 1974). Other researchers propose that crescent membranes are derived from the ER-Golgi intermediate compartment (ERGIC) (Sodeik *et al.*, 1993; Griffiths *et al.*, 2001a; Griffiths *et al.*, 2001b). This theory is strengthened by

<u>CORE</u>	<u>IMV MEMBRANE</u>	<u>EEV MEMBRANE</u>
A3L	A13L	A33R
A4L	A14L	A34R
A10L	A17L	A36R
A12L	A27L	A56R
A30L	D8L	B5R
D2L	D13L	F12L
D3R	G4L	F13L
E8R	H3L	
F17R	H5R	
G7L	I5L	
I7L*	L1R	
J1R		
L4R		

Table 3. Non-enzymatic virion components. Reported non-enzymatic vaccinia virion components are listed. They are grouped into three classes: those found in the core, those found in the intracellular mature virion (IMV) membrane, and those found in the extracellular enveloped virion (EEV) membrane.

* While I7L was initially reported as a non-enzymatic structural component (Kane and Shuman, 1993; Moss, 1996), it has recently been shown to possess proteinase activity (Byrd *et al*, 2003).

the observation that several viral membrane proteins, namely A13L, A14L and A17L, localize to the ERGIC prior to crescent formation (Salmons *et al.*, 1997; Krijnse-Locker *et al.*, 1996). Proponents of the *de novo* synthesis model argue that the complete lack of cellular markers in the IV membrane is inconsistent with this model.

The structure of the crescent membranes has also become a hotly debated issue. Recent evidence suggesting the "single" bilayer is actually two tightly apposed bilayers has led support to the idea that cisternae from the ER-Golgi membrane system might provide membrane precursors for crescent formation (Sodeik *et al.*, 1994, Roos *et al.*, 1996). It was found that treating infected cells with a protease for a very short time caused dramatic changes in virion morphology, with the bold single membrane appearing to separate into two closely associated membranes (Roos *et al.*, 1996). Other groups have observed similar structures but have provided a different interpretation. They propose that the two layers seen are from a single lipid bilayer that has peeled away from the overlying layer of proteinacious spicules (Hollinshead *et al.*, 1999).

1.5.6 Vaccinia Virus Morphogenesis II - Core Formation

To date, 12 viral proteins have been shown to comprise the structural component of the IV core (Sarav and Joklik, 1972; Jensen *et al.*, 1996; Pedersen *et al.*, 2000) (Table 3). Four of the most abundant virion core proteins are A3L, A4L, A10L, and L4R (Sarav and Joklik, 1972; Jensen *et al.*, 1996). A3L (Pedersen *et al.*, 2000), A4L (Maa and Esteban, 1987) and A10L (Van Meir and Wittek, 1988) form the outer proteinacious shell of the core. L4R, comprising 11% of dry virion weight (Yang and Bauer, 1988), is found in the core interior which binds viral genomic DNA and keeps it in a transcription-competent state (Bayliss and Smith 1997; Pedersen *et al.*, 2000).

1.5.7 Vaccinia Morphogenesis III – Maturation into Intracellular Mature Virions (IMV)

Within the IV, several morphological changes take place. Electron microscopic studies have shown condensation of viral genomic material, and gel electrophoresis of virion proteins reveals that several proteolytic cleavages occur within the virion during maturation to yield an intracellular mature virion (IMV) (Moss and Rosenblum, 1973; Heljasvaara *et al.*, 2001). The protein composition of the IMV membrane has been characterized by several independent groups (Ichihashi *et al.*, 1984; Takahashi *et al.*, 1994; Jensen *et al.*, 1996). Eleven IMV membrane proteins were identified: A13L, A14L, A17L, A27L, D8L, D13L, G4L, H3L, H5R, I5L, and L1R. Of these IMV membrane components, 10 have been further characterized through subsequent studies. Three of the most abundant IMV membrane proteins are A13L (Salmons *et al.*, 1997), A14L (Salmons *et al.*, 1997) and A17L (Rodriguez *et al.*, 1995). All three are integral membrane proteins that are co-translationally inserted into the ER and transported to the ERGIC, prior to their eventual incorporation into nascent virions (in support of the hypothesis that crescent membranes are derived from ERGIC precursors). Inducible knockouts of A14L and A17L have been characterized, with the resulting phenotypes displaying several-fold drops in viral titres *in vitro* and a disruption in early stages of IMV formation (Rodriguez *et al.*, 1996; Rodriguez *et al.*, 1998). The possible cell-adhesion protein, A27L, is not anchored into IMV membranes via a transmembrane helix, but is rather targeted to the IMV membrane through direct interactions with A17L (Rodriguez *et al.*, 1996; Chung *et al.*, 1998). The cell-adhesion molecule, D8L, is an integral membrane protein possessing a transmembrane stretch at its C-terminus (Hsiao *et al.*, 1999). H3L is post-translationally inserted into IMV membranes via a hydrophobic C-terminal anchor (da Fonseca *et al.*, 2000a), and has been shown to be important for both adsorption of IMV to mammalian cells and for early stages of virion morphogenesis

(da Fonseca *et al.*, 2000b; Lin *et al.*, 2000). L1R is required for virion morphogenesis (Ravanello *et al.*, 1994a; Ravanello *et al.*, 1994b), and efficient disulphide bond formation in L1R is dependent upon a second IMV membrane protein, G4L, which acts as a redox intermediate in the vaccinia disulphide bond formation pathway (White *et al.*, 2002; Senkevich *et al.*, 2002). D13L is responsible for the formation of spicules on the outer surface of the crescent membranes (Mohandas and Dales, 1995) while H5R has been identified as a viral transcription factor (Beaud *et al.*, 1995; Beaud and Beaud, 1997; Kovacs and Moss, 1996). Of the 11 IMV membrane proteins identified only I5L remains uncharacterized. I5L is 79 amino acids and is extremely hydrophobic. It possesses a hydrophobic C-terminus similar to membrane anchors found in D8L and H3L, suggesting it may target to viral membranes in a similar fashion.

1.5.8 Vaccinia Morphogenesis IV – Intracellular Enveloped Virion (IEV) Formation

Upon maturation from IV, IMV are fully infectious and can be released from the infected cell upon cell lysis (Ichihashi, 1996; Vanderplasschen *et al.*, 1997), however a small proportion of IMV can be further wrapped by two additional bilayers derived from the trans-Golgi compartment to form intracellular enveloped virions, or IEV (Hiller and Weber, 1985; Schmelz *et al.*, 1994). The IEV thus possess 3 or 4 bilayers, depending on whether the IMV forms from a single or double membrane. IEV are then transported to the cell periphery where fusion with the plasma membrane results in loss of the outermost bilayer and the release of the virion into the extracellular space (Payne, 1980; Smith *et al.*, 2002). The remaining Golgi-derived membrane contains several viral proteins not found in IMV (Payne, 1978; Lorenzo *et al.*, 2000) (Table 3). Some of the extracellular virions remain associated with the plasma membrane as cell-associated enveloped virus (CEV) or are released from the cell surface as extracellular enveloped virions (EEV). CEV and EEV are thought to mediate cell-to-cell (Blasco and Moss, 1992) and long

range transmission (Payne, 1980), respectively, but are otherwise indistinguishable from one another both visually and biochemically.

The mechanism of CEV infection of neighboring cells is reminiscent of *Listeria* spp. (Tilney and Portnoy, 1989) and *Shigella* spp. (Bernardini *et al.*, 1989) bacteria. The CEV particles induce the polymerization of actin at their base. The growing actin filament slowly propels the CEV away from the cell on a structure that has been termed an actin tail (Cudmore *et al.*, 1995). These actin tails are presumed to bring CEV into direct physical contact with neighboring cells, thus promoting cell-to-cell spread of the infection (Cudmore *et al.*, 1995; Rottger *et al.*, 1999; Scaplehorn *et al.*, 2002).

The family of EEV-specific membrane proteins contains 7 identified members: F12L, F13L, B5R, A33R, A34R, and A36R and A56R. F13L and B5R are necessary for the correct wrapping of IMV to form IEV (Blasco and Moss, 1991; Rottger *et al.*, 1999; Husain *et al.*, 2001). F12L mutants are unable to efficiently transport IEV to the cell-surface (van Eijl *et al.*, 2002). A33R, A34R and A36R knockouts, although still able to transport fully wrapped IEV to the cell surface, are unable to induce the formation of actin tails (Wolffe *et al.*, 1997; Wolffe *et al.*, 1998; Roper *et al.*, 1998). More specifically, A33R has been shown to facilitate the recruitment of A36R to the IEV membrane (Wolffe *et al.*, 2001), where it subsequently becomes phosphorylated. Once phosphorylated by Src family kinases, A36R recruits the Nck adaptor protein and N-WASP to the base of the CEV, both of which are cellular factors necessary for actin polymerization (Frischknecht *et al.*, 1999).

1.6 Insertion of Poxviral Proteins into Membranes

Poxviral protein targeting to sub-cellular organelles is an active area of research, with much of this work relating directly to how viral proteins are trafficked within the host cell and how they reach their final destinations in the virion particle. Many

trafficking studies have been carried out with vaccinia IMV proteins in order to determine how they become associated with nascent IMV particles. For poxviral membrane proteins, at least two different types of membrane targeting have been described: classical co-translational membrane insertion utilizing the signal recognition particle pathway (Krijnse-Locker *et al.*, 1996; Wilcock *et al.*, 1999), and post-translational insertion characteristic of tail-anchored proteins (da Fonseca *et al.*, 2000; Husain *et al.*, 2003). Co-translational examples include A13L (Salmons *et al.*, 1997), A14L (Salmons *et al.*, 1997) and A17L (Rodriguez *et al.*, 1995), while post-translational examples include H3L (da Fonseca *et al.*, 2000) and F13L (Husain *et al.*, 2003). It is interesting to note however, that regardless of which route is taken, none of these proteins show evidence of signal peptide cleavage, N-glycosylation or any other signature of ER trafficking. This has led to the hypothesis that some of these proteins may associate with nascent virions via unconventional pathways (da Fonseca *et al.*, 2000).

1.7 Classical Co-translational Protein Insertion into Membranes

All organisms, both prokaryotes and eukaryotes, must deal with the problem of how to transport newly synthesized proteins across membranes. A significant proportion of proteins are membrane-bound, contained within membrane-bound organelles, or are secreted from the cell. All of these destinations require translocation across a membrane. Eukaryotic cells have developed a complex protein translocation system to deal with this problem: the signal-recognition particle (SRP) pathway (reviewed by Walter and Johnson, 1994).

Although slightly different versions of this pathway are present in different organelles, all follow four basic steps: (1) recognition of a signal sequence on the protein to be targeted, (2) transport of the protein to the target membrane, (3) engagement of the

translocation machinery, (4) translocation across the membrane or integration of the polypeptide into the lipid bilayer.

The first step is the recognition of a signal sequence on the protein to be targeted. A signal sequence is essentially an N-terminal hydrophobic segment of 6-12 hydrophobic amino acids, often with a basic residue immediately preceding the hydrophobic stretch (von Heijne, 1985). This region is predicted to be α -helical, but no obvious sequence conservation exists between different signal sequences, suggesting that hydrophobicity of the N-terminus is the critical factor (Walter and Johnson, 1994). As a cytosolic ribosome begins to translate a nascent polypeptide, the polypeptide begins to emerge from the surface of the ribosome. A soluble complex of proteins, collectively referred to as the 'signal recognition particle', scans the sequences emerging from cytosolic ribosomes, looking for polypeptides that possess a signal sequence (Walter *et al.*, 1981; Walter and Blobel, 1981a). Binding of the SRP to ribosomes displaying a signal sequence causes a translation arrest (Walter and Blobel, 1981b). The ribosome/nascent chain/SRP complex is often referred to as the 'targeting complex' (Walter *et al.*, 1981).

The second step involves the transport of the targeting complex to the target membrane (Walter and Blobel, 1981b). For the vast majority of such proteins, the target membrane is the endoplasmic reticulum. The SRP component of the targeting complex binds to the SRP receptor, an integral ER membrane protein (Gilmore *et al.*, 1982a; Gilmore *et al.*, 1982b). The third step in co-translational insertion namely engagement of the translocation machinery, is initiated by GTP hydrolysis. This results in dissociation of SRP from the targeting complex and transfer of the ribosome to an ER membrane complex, the translocon (Walter and Blobel, 1980; High *et al.*, 1993; Oliver *et al.*, 1995). The absence of SRP allows translation to continue. The fourth step is translocation across the membrane, which occurs co-translationally. It has been shown that the growing polypeptide remains in an aqueous environment during the entire translocation process.

(Gilmore and Blobel, 1985). It is presumed that the translocon forms a gated aqueous pore through which the polypeptide traverses en route to the ER lumen (Gilmore and Blobel, 1985).

Some proteins are completely translocated into the ER lumen, such as those destined for secretion, while others remain anchored in the membrane as either type I or type II integral membrane proteins, depending on their final membrane topology. Type I membrane proteins are translocated N-terminus first with the resulting orientation having the N-terminus in the lumen and the C-terminus in the cytosol. Type II transmembrane proteins lack N-terminal signal sequences. They instead possess a signal sequence in an internal region of the polypeptide (High *et al.*, 1991). During translation, this internal signal sequence is recognized by the SRP and translation is halted in the normal fashion. It is hypothesized that at the translocon, a loop of the nascent chain, which includes the signal sequence, is initially inserted into the gated channel. Translation resumes during which the C-terminal region is translocated while the N-terminal region is held at the pore much like a stop-transfer sequence. The resulting orientation having the N-terminus in the cytosol and the C-terminus in the lumen with the internal signal sequence serving as the transmembrane anchor.

1.8 Tail-Anchored Proteins

There is a small, but expanding family of integral membrane proteins which have their membrane anchor at the C-terminus (reviewed by Kutay *et al.*, 1993; Wattenberg and Lithgow, 2001; Borgese *et al.*, 2003). To date, only a few dozen tail-anchored proteins have been described. They are found in organisms as diverse as bacteria, yeast, mammals and viruses (Table 4). As well, the intracellular destinations are also quite diverse with examples described for virtually every intracellular organelle and membrane system. Most of these proteins do not possess a signal sequence. Moreover, their

Localization/Trafficking Proteins	Localization	References
Vamp-1	ER/M	Kim <i>et al.</i> , 1997
Sec61 β /Sec61 γ	ER	Wattenberg and Lithgow, 2001
Sec12	ER	Barlowe and Schekman, 1993; Wattenberg and Lithgow, 2001
SRPR α	ER	Wattenberg and Lithgow, 2001
Tom5, Tom6, Tom7, Tom22	M	Horie <i>et al.</i> , 2002; Dembowski <i>et al.</i> , 2001; Allen <i>et al.</i> , 2002; Saeki <i>et al.</i> , 2000; Kanaji <i>et al.</i> , 2000; Horie <i>et al.</i> , 2002
OMP25	M	Rose and Fink, 1987;
Kar1	NE	Kutay <i>et al.</i> , 1993
Syntaxin	PM	Jantti <i>et al.</i> , 1994
Enzymes and Cofactors		
Cytochrome b5	ER/M	Honsho <i>et al.</i> , 1998; Pedrazzini <i>et al.</i> , 2000
Heme oxygenase	ER	Yoshida and Sato, 1989
Cytochrome P450	ER	Wattenberg and Lithgow, 2001
Aldehyde dehydrogenase	ER	Masaki <i>et al.</i> , 1994
Monoamine oxidases	M	Wattenberg <i>et al.</i> , 2001
Pig-A	ER/G	Wattenberg <i>et al.</i> , 2001
Ubc6	ER	Walter <i>et al.</i> , 2001
Hepatitis C RNA polymerase	ER	Schmidt-Mende <i>et al.</i> , 2001; Ivashkina <i>et al.</i> , 2002
Polyoma virus middle-T antigen	ER/PM	Hofer <i>et al.</i> , 1995
Peroxisomal ascorbate peroxidase	P	Mullen and Trelease, 2000
Other		
Bcl-2 and family members	ER/M	Chen-Levy and Cleary, 1990; Kim <i>et al.</i> , 1997
Vaccinia virus H3L	VF	da Fonseca <i>et al.</i> , 2000
Pseudorabies virus Us9	G	Brideau <i>et al.</i> , 1998
Herpes simplex virus-2 UL34	ER	Shiba <i>et al.</i> , 2000
Herpes simplex virus-2 UL56	G	Koshizuka <i>et al.</i> , 2002
Epstein-Barr virus BHRF-1	M	Khanim <i>et al.</i> , 1997; Kutay <i>et al.</i> , 1993

Table 4. Localizations of a range of tail-anchored proteins.

A range of tail-anchored proteins have been classified into three groups based on their putative or demonstrated functions. Their respective intracellular localizations are also indicated: endoplasmic reticulum (ER), Golgi compartment (G), mitochondria (M), nuclear envelope (NE), peroxisome (P), plasma membrane (PM), and viral factory (VF).

membrane anchor is generally found within the last 50 amino acids. Since at least 60 amino acids must be translated following the signal sequence until enough of the nascent chain has emerged from the ribosome to allow for SRP recognition (Walter and Blobel, 1981b), tail-anchored proteins should not be accessible to the SRP complex and must thus target via a novel pathway. A further implication of the C-terminal anchor is that insertion is obligatorily post-translational event, since translation would have to be completed before the C-terminal anchor emerges from the ribosome.

The C-terminal transmembrane segment is generally 12 to 22 amino acids in length and is presumed to be helical. There is usually a very short stretch of amino acids (<10) following the transmembrane domain which sometimes contains positively charged residues important for targeting (Kuroda *et al.*, 1998). This is especially true for proteins destined for the mitochondria (Horie *et al.*, 2002). It is well known that the length of transmembrane domains is an important factor in determining membrane selectivity (Whitley *et al.*, 1996; Watson and Pessin, 2001) since different membranes have different thicknesses. Mitochondria possess the thinnest intracellular membrane, after which the ER, Golgi compartment, and plasma membrane have thicker membranes respectively (Pelttari and Helminen, 1979). In some cases other features of the tail are important for membrane targeting such as key residues in the anchor itself, or in adjacent regions (Rayner and Pelham, 1997; Honsho *et al.*, 1998).

Most tail-anchored proteins are targeted to the mitochondria or endoplasmic reticulum membrane systems. Although tail-anchored proteins can be found in other intracellular membranes such as the nuclear envelope, Golgi compartment, peroxisomes, endosomes and plasma membrane, it has been suggested that these proteins are first targeted to the endoplasmic reticulum and are subsequently shipped to these other membranes using vesicular traffic (Wattenberg and Lithgow, 2001).

Although members of the tail-anchored protein family are found in a wide range of cellular pathways, a disproportionate number seem to either comprise membrane insertion machinery or mediate the ensuing transport of proteins throughout the cell (Wattenberg and Lithgow, 2001). For example, the SNARE proteins which include synaptobrevins, Vamps and syntaxins are tail-anchored and involved in vesicle-mediated trafficking (Bennett, 1995). Sec61 β and Sec61 γ , the major components of the ER translocon, are also tail-anchored (Wattenberg and Lithgow, 2001), as are components of the mitochondrial translocon, or TOM complex (Allen *et al.*, 2002; Wattenberg and Lithgow, 2001).

In mammalian systems at least two distinct post-translational insertion pathways have been described (Kim *et al.*, 1997; Kim *et al.*, 1999). One route is ATP-dependent, saturable and inhibited by a light trypsin treatment of microsomes, which is strong evidence of a receptor-mediated process at the target membrane (Kim *et al.*, 1997; Kim *et al.*, 1999). The other route is ATP-independent, non-saturable and not inhibited by pre-treatment of microsomes with trypsin, suggestive of spontaneous insertion of these tail-anchored proteins directly into microsomes (Kim *et al.*, 1997). Vamp-1, a member of the SNARE protein family, demonstrates the former route (Kim *et al.*, 1999; Teng *et al.*, 2001), while Cb5 and Bcl-2 display the latter (Kim *et al.*, 1997).

1.9 A Search for Tail-anchored Proteins in Vaccinia

The complete genome sequence of vaccinia virus has been delineated (Goebel *et al.*, 1990). To identify possible tail-anchored proteins in vaccinia virus we performed a search of vaccinia virus open reading frames looking for proteins which possessed a stretch of 12-24 hydrophobic amino acids flanked by positive charges, with 10 or fewer residues immediately following the hydrophobic stretch (Figure 4). Four proteins were identified that fit this profile: B6R, D8L, F1L and I5L. All except D8L were completely

C-terminal Pattern Search Query

[KRH] [GAVLISCTMPFYEW](12,24) [KRH] X(1,10)

Vaccinia Virus

- B6R **KLYFYASLTTIGTIAGGVCYYLLMHLVSLYK**
- D8L **KTFAIIAIVFVFILTAILFLMSRRYSREKQN**
- I5L **KYIGLFIYIPGTIILYATYVKSLLMKS**
- F1L **KLIGITAIMFATYKTLKYMIG**

Figure 4. Search parameters for vaccinia virus tail-anchored proteins. The search query used to search the ~200 open reading frames of the vaccinia virus strain Copenhagen genome is shown. The four positive hits obtained by the search are also shown, along with their respective hydrophobic C-termini.

uncharacterized. D8L has been shown to localize to the IMV outer membrane where it is involved in cell adhesion (Hsiao *et al.*, 1999).

Studies in our laboratory have shown that a B6R-green fluorescent protein (GFP) fusion localizes to the endoplasmic reticulum in uninfected, transiently transfected HeLa cells (Figure 5, panels D-F), while a GFP control displays a diffuse staining pattern (Figure 5, panels A-C). Deletion of the C-terminal hydrophobic tail resulted in a GFP fusion that completely lost its ability to localize to the ER (Figure 6, panel A-C), and instead displayed a soluble GFP-like distribution (Figure 5, panel B). Likewise, other studies from our lab have shown that a GFP-FIL fusion localizes to the mitochondria (Figure 7, panels D-F), as evidenced by its colocalization with the mitochondria-specific fluorescent dye, MitoTracker (panel F). As with B6R, deletion of the hydrophobic tail of FIL results in a GFP fusion with a diffuse staining pattern (Figure 8, panel A-C) indistinguishable from a GFP control (Figure 7, panels A-C). In this regard, both B6R and FIL behave as tail-anchored proteins.

It is intriguing that B6R, FIL and D8L all possess similar C-terminal anchoring domains, yet target to completely different subcellular sites during infection. For this reason, it would be both interesting and informative to determine if I5L is a tail-anchored protein and to which cellular structure it localizes. Such studies will not only shed light on how this unusual class of proteins can achieve such highly specific and highly variable subcellular targeting, but also on viral protein trafficking and virion assembly in general.

The only reference to I5L in poxviral literature is that it can be found in the IMV membrane following extraction of this membrane with detergent (Takahashi *et al.*, 1994). This group identified I5L by N-terminally sequencing IMV proteins separated by gel electrophoresis, and sequencing a peptide corresponding to the extreme N-terminus of I5L. The I5L gene encodes for a hydrophobic 79-amino acid polypeptide with an

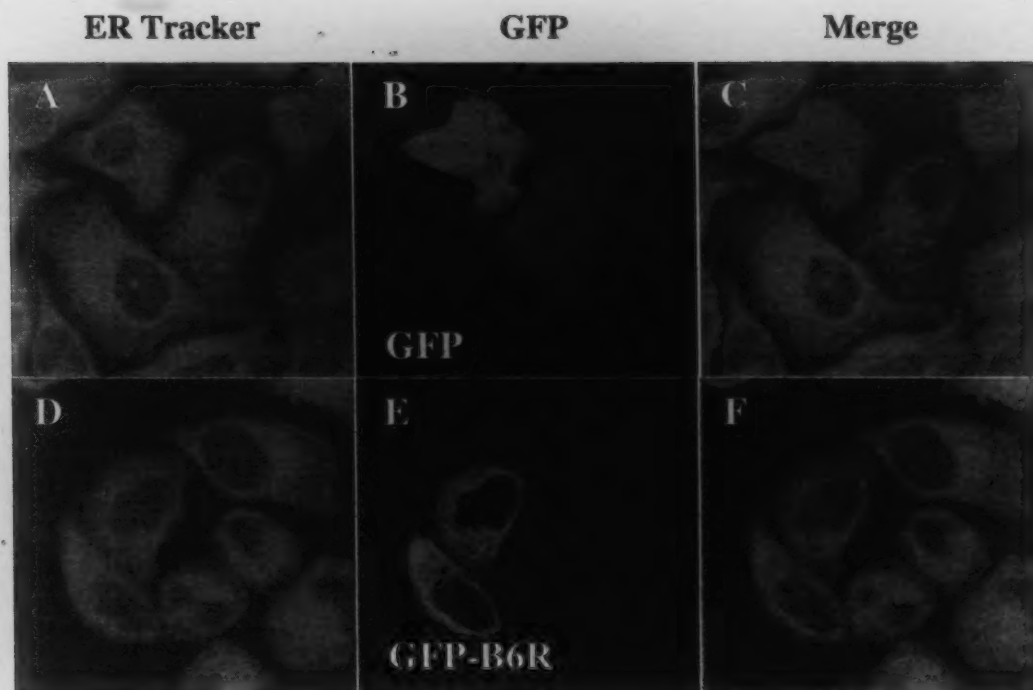


Figure 5. The vaccinia B6R protein localizes to the endoplasmic reticulum. HeLa cells were transiently transfected with GFP or a GFP-B6R fusion, in which GFP was fused to the N-terminus of B6R. Live cells were stained with ER Tracker and analyzed by confocal microscopy. Two separate images were obtained for each cell: the ER Tracker emission (panels A and D) which stains endoplasmic reticulum membranes, and GFP emission (panels B and E). A merged image of ER Tracker and GFP are provided (panels C and F). Images courtesy of Shawn Wasilenko.

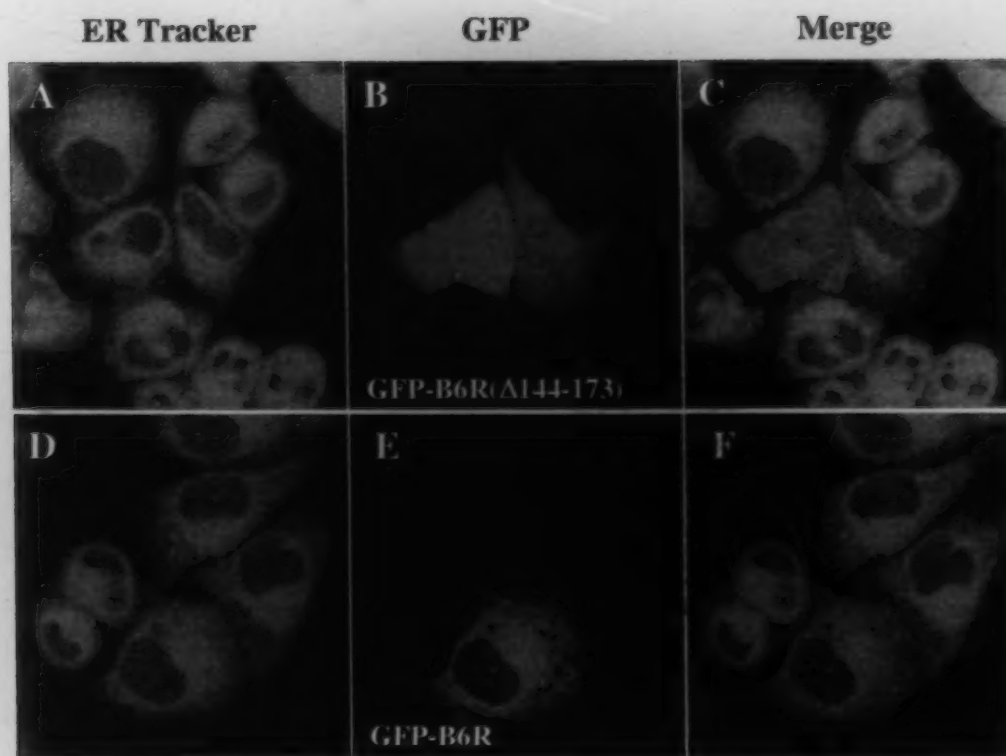


Figure 6. The hydrophobic tail of B6R is necessary for ER targeting. HeLa cells were transiently transfected with either a full-length GFP-B6R fusion (panels D-F) or a C-terminally truncated GFP-B6R fusion in which the C-terminal 30 amino acids of B6R were deleted (panels A-C). Live cells were stained with ER Tracker, a fluorophore which localizes to the endoplasmic reticulum, and analyzed by confocal microscopy. Two separate images were obtained for each cell: the ER Tracker emission (panels A and D), and GFP emission (panels B and E). A merged image of ER Tracker and GFP are provided (panels C and F). Images courtesy of Shawn Wasilenko.

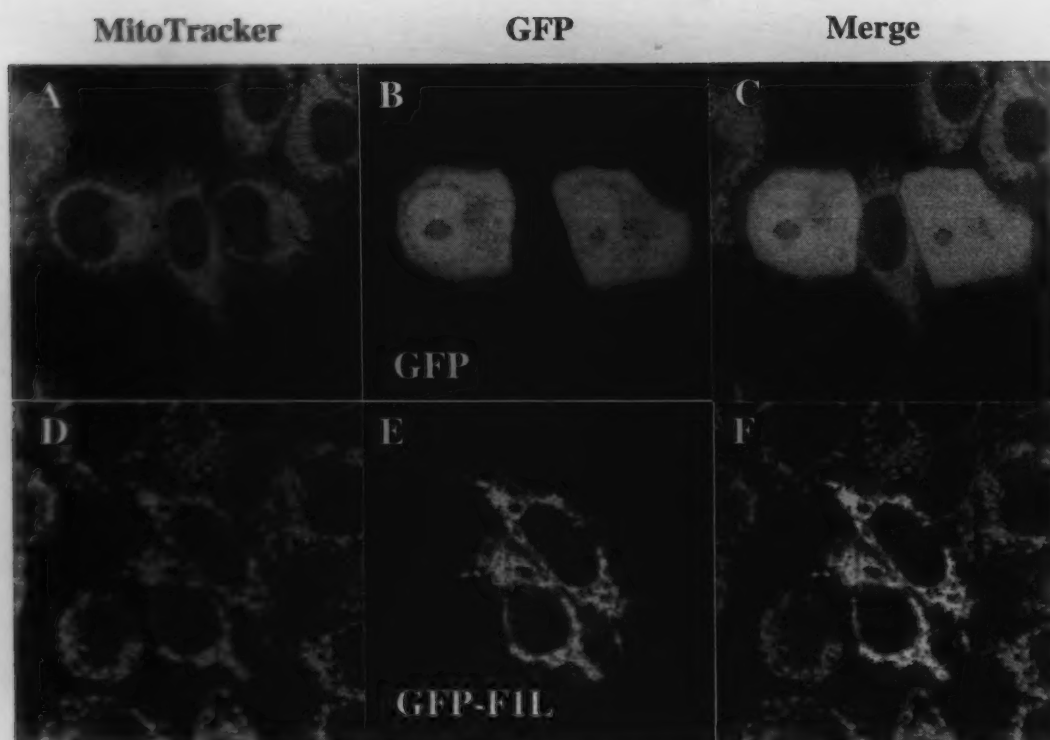


Figure 7. The vaccinia F1L protein localizes to the mitochondria. HeLa cells were transiently transfected with GFP or a GFP-F1L fusion, in which GFP was fused to the N-terminus. Live cells were stained with MitoTracker and analyzed by confocal microscopy. Two separate images were obtained for each cell: the MitoTracker emission (panels A and D) which stains mitochondria specifically, and GFP emission (panels B and E). A merged image of MitoTracker and GFP are provided (panels C and F). Images kindly provided by Tara Stewart.

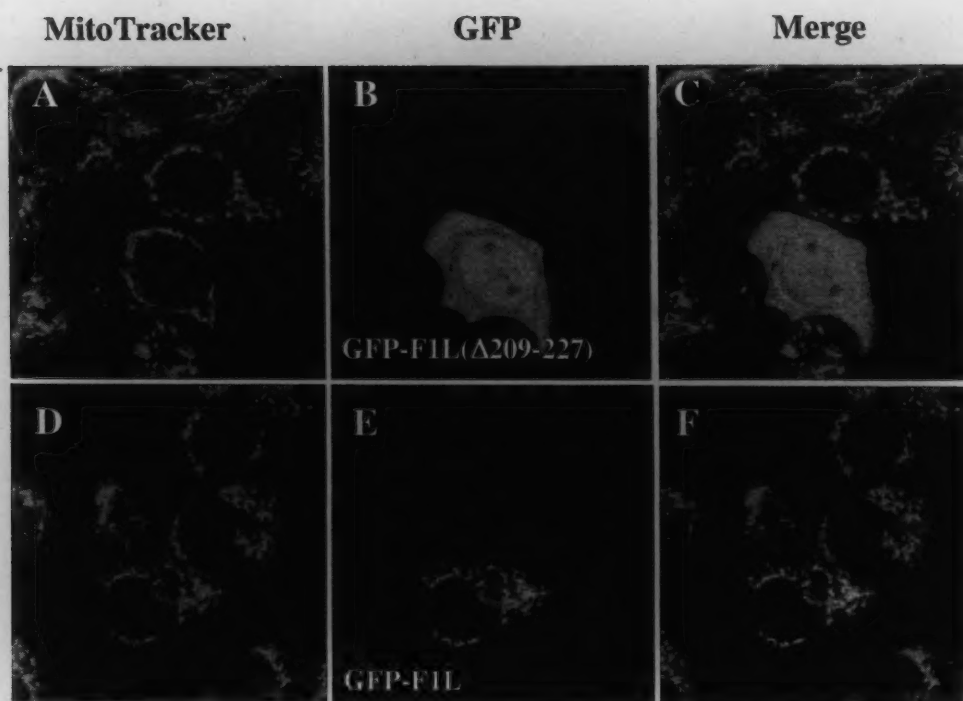


Figure 8. The hydrophobic tail of F1L is necessary for targeting to mitochondria. HeLa cells were transiently transfected with either a full-length GFP-F1L fusion (panels D-F) or a C-terminally truncated GFP-F1L fusion in which the C-terminal 19 amino acids of F1L were deleted (panels A-C). Live cells were stained with MitoTracker, a mitochondria-specific fluorophore, and analyzed by confocal microscopy. Two separate images were obtained for each cell: the MitoTracker emission (panels A and D), and GFP emission (panels B and E). A merged image of MitoTracker and GFP are provided (panels C and F). Images kindly provided by Tara Stewart.

expected size of 9-kDa. A feature which distinguishes I5L from D8L, B6R or F1L, is the presence of an N-terminal hydrophobic stretch.

A sequence alignment of I5L was generated using orthologues found during a Blast search of NCBI sequences (Figure 9). Approximately a dozen obvious matches were obtained during this search, all of which were from I5L orthologues in other members of the *Poxviridae* family. Upon visual analysis of the alignment it is immediately apparent that I5L is highly conserved throughout the *Poxviridae*. Although sequence similarity is high for even the most divergent orthologues, the division between the *Orthopoxviridae* and other genera is clearly evident. Amongst the orthopoxviruses (upper 6 sequences) sequence identity approaches 100%. Amongst the other genera (lower 6 sequences), sequence identity ranges from 50-85%, while sequence similarity ranges from 75-95%. Sequence identity between orthopoxvirus I5L orthologues and those from the other poxviral genera is approximately 40%, while sequence similarity is close to 70%. Such high conservation between distantly related poxviruses suggests that I5L is an important viral protein. General features of the sequence are also conserved, specifically the presence of two terminal hydrophobic stretches bifurcated by a short polar region. Both hydrophobic regions are predicted to be alpha helical. The hydrophobic termini are the most highly conserved regions with the intervening region being less so. Analysis with algorithms did not reveal any glycosylation, palmitoylation or myristoylation sites or any other modification that would be indicative of membrane anchoring or passage through the endoplasmic reticulum.

Following the C-terminal hydrophobic stretch is a short polar tail of roughly 7 amino acids. Such tails have been shown to be important for the correct localization of several tail-anchored proteins, especially those destined for the mitochondria (Kuroda *et al.*, 1998; Horie *et al.*, 2002). The importance of this region to I5L localization was also of interest to us.

At the outset, the two hypotheses which guided the early stages of my thesis were as follows: (1) I5L is a tail-anchored protein and (2) I5L is an essential viral protein, most likely involved in virion morphogenesis. The basis for these hypotheses include (1) the presence of a tail-anchoring motif in I5L, (2) the reported presence of I5L in the virion particle (Takahashi *et al.*, 1994), (3) the presence of I5L in the central region of the poxviral genome which is rich in 'housekeeping' genes, (4) the presence and high sequence conservation of I5L in all vertebrate poxviruses, (5) the lack of an obvious catalytic motif, (6) the fact that I5L is under the control of a late promoter, a common feature of virion structural proteins, and (7) the high hydrophobicity of I5L, another common feature of virion structural proteins. Although the choice of how to proceed was influenced by the results of each successive experiment, attempts to test these hypotheses remained the central driving force behind my work.

With the aforementioned hypotheses as a guide, the objectives of my thesis were as follows: (1) To determine the localization of I5L both in uninfected cells and in the context of infection, (2) to determine the region(s) of I5L that are important for intracellular targeting, (3) to elucidate the mechanisms of membrane targeting (if such targeting occurs), and most importantly (4), to determine the function of I5L during infection.

2. Materials and Methods

2.1 Cell Lines

Baby green monkey kidney (BGMK) cells, a gift from Dr. S. Dales, were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% newborn calf serum (both from Invitrogen Life Technologies, Inc.), 50 U of penicillin/mL (Invitrogen Life Technologies, Inc.), 50 µg of streptomycin/mL (Invitrogen Life Technologies, Inc.) and 2 mM glutamine (Invitrogen Life Technologies, Inc.). HeLa (American Type Tissue Culture) and baby hamster kidney (BHK-21) cells (kindly provided by Dr. Tom Hobman, University of Alberta) were grown in DMEM supplemented with 10% heat inactivated fetal bovine serum (FBS) (Invitrogen Life Technologies, Inc.), 50 U of penicillin/mL, 50 µg of streptomycin/mL and 2 mM glutamine.

2.2 Viruses

A recombinant vaccinia virus strain Copenhagen expressing β -galactosidase (VV65) and a recombinant vaccinia virus strain Western Reserve also expressing β -galactosidase (VV601) were gifts from Dr. G. McFadden (Robarts Research Institute, London, Ontario, Canada). Wildtype ectromelia virus (EV) strain Moscow was kindly provided by M. Buller, St. Louis, MO. All viruses were routinely propagated in BGMK cells and isolated as previously described (Stuart *et al.*, 1991).

2.3 Purification of Viral DNA

Purified viral DNA was prepared for use in molecular biology applications. 1×10^6 BGMK cells were infected at a multiplicity of infection (MOI) of 1. Initial infections were carried out for 1 hr at reduced volume with periodic rocking. Infected cells were incubated for a further 16 hours at 37°C, 5% CO₂. The infected cells were harvested

using trypsin, and resuspended in 200 μ L of lysis buffer containing 5 mM Tris pH 8.0, 2.25% Tween-20 (Fischer Biotech Inc., Fair Lawn NJ, USA), 2.25% NP-40 (Sigma Chemical Company, St. Louis, MO, USA) and 250 μ g/mL proteinase K (Sigma Chemical Co.). Lysis reactions were incubated at 55°C for 4 h with periodic inversion. Samples were then boiled for 10 min followed by extraction with 400 μ L of a 1:1 phenol:chloroform mixture. The aqueous phase was then precipitated with 2.5 volumes ice-cold 95% ethanol. DNA was resuspended in 100 μ L deionized water and treated with 100 μ g RNase (Sigma Chemical Co.) for 10 min at 37°C.

2.4 Cloning of ectromelia virus I5L orthologue

Primers 5'-GAATTCATGGTGGATGCTATAACC and 5'-GGATCCACTTTTCATTAATAGGG, which were originally designed for amplification of the vaccinia strain Copenhagen I5L gene, were used to amplify the I5L orthologue from EV strain Moscow. Amplification of EV I5L was performed by polymerase chain reaction (PCR). Triplicate reactions were performed, with each replicate cloned and sequenced independently. Each PCR reaction contained 1X High Fidelity PCR buffer (Invitrogen Life Technologies, Inc.), 1.25 mM magnesium chloride (Invitrogen Life Technologies, Inc.), 200 μ M deoxynucleotide mixture (Invitrogen Life Technologies, Inc.), 250 nM forward primer, 250 nM reverse primer, 100 ng purified EV DNA template, and 5 U High Fidelity *Thermus aquaticus* polymerase (Invitrogen Life Technologies, Inc.). PCR conditions were a 2 min denaturation at 95°C, followed by 30 amplification cycles (30 sec at 95°C, 30 sec at 55°C, 30 sec at 72°C) and concluded with a 10 min elongation at 72°C. PCR was carried out using a Techne thermal cycler. The PCR fragment generated from each replicate reaction was cloned into the TA-Cloning vector, pGEM-T (Promega Corporation), to generate the pGEM-T/I5L-EV.1, pGEM-

- 1) 15L-FW: GAATTCATGGTGGATGCTATAACC
- 2) 15L-RV: GGATCCACTTTTCATTAATAGGG
- 3) 15LNT-RV: GGATCCAATCGTTACGGCTCGATT
- 4) 15LNS-FW: GAATTCATGATAGTCAAGGAGTTA
- 5) 15LNC-RV: GAATTCATGGTGGATGCTATAACC
- 6) 15LT-FW: GAATTCATGGTAACGATTTTCAAATAT
- 7) 15L-RVS: GGATCCTAACTTTTCATTAATAG
- 8) 15LTM-F: GAATTCGTAACGATTTTCAAATAT
- 9) 15LTM-R: GGATCCTTATGTAGCGTACAAAATTAT
- 10) 15LNTS-R: GGATCCTAAATCGTTACGGCTCGATTAAAC
- 11) 15LTNT-F: GAATTCACCATGGTGGATGCTATAACC
- 12) 15LTNT-NSF: GAATTCACCATGGTAGTCAAGGAGTTA
- 13) 15LTNT-NTR: GGATCCTAAATCGTTACGGCTAGATT
- 14) 15LKO-F1: TGTAAGAAACATTGTTTCGTC
- 15) 15LKO-R1: GATCCAGATCTGCATTAGAAATTACCATCAAAAG
- 16) 15LKO-F2: AATGCAGATCTGGATCTTTATCTATATACCAGGA
- 17) 15LKO-R2: TGATTTTATCAAACATGACAT
- 18) 15LNS2-F: GAATTCATGCAATCATTAAAGTTT
- 19) 15LHAKO-R: GGATCCTCAAGCATAGTCTGGAACATCATATGGATAACTTTTCATTAATAGGGATTG
- 20) 15LHA-F: GTCGAC ATGTACCCATACGACGTCCTCCAGACTACGCTATGGTG GATGCTATAACC
- 21) 15LST-F: GAATTCATGGTGGATGCTATAACCGTT
- 22) 15LST-R: CTCGAGTAACTTTTCATTAATAG
- 23) 15LHT-F: CATATGTGGATGCTATAACCGTT
- 24) 15LRF-F: AAGCTTCCTTTCCGACGCCCAAGACGT
- 25) 15LRF-R: GTCGACCATCAAAAGCATTAAATACAGT
- 26) 15LLF-F: GCGGCCGCATACCAGGAACGATAATTTTG
- 27) 15LLF-R: AGATCTTAACTATATCCATGGTGATAC
- 28) 15L-F2: GTCGACACCATGGTGGATGCTATAACC
- 29) HAKO-F: CACAATTAAATCATTGACTGCT
- 30) HAKO-R: AGTGTATGTGACGGTGTCTGT
- 31) B6RTNT-F: GAATTCACCATGGCCATGTCTTCTTCAGTGGAT
- 32) B6RTNT-R: GGATCCTATTATACAACTAACTAG
- 33) D8L-F2: CTCGAGATGCCGCAAACTATCT
- 34) D8L-R2: GAATTCCTAGTTTGTCTTCTCG
- 35) A45R-F: CTCGAGATGGCTGTTGTATAATA
- 36) A45R-R: CTCGAGTCAAACGCCATTCTCGTT
- 37) A40RKO-F: AATGCAGATCTGGATCGGATATGGGGTAGGAGAA
- 38) A40RKO-R: GATCCAGATCTGCATTTCATCTTTATCGTTTT
- 39) A40R-FW: ATGAACAAACATAAGACAGA
- 40) A40CO-RV: CTAACCGAAGTAGTGGTATG
- 41) A40WR-RV: TTATATAGTGTAAACACGAATG

Table 5. List of primers. All primers used during the course of the thesis are numbered and listed. Primers are arranged in 5'-to-3' orientation. Restriction sites are underlined. Primers 19 and 20 also possess sequences encoding a hemagglutinin antigen tag, which are italicized.

T/I5L-EV.2, and pGEM-T/I5L-EV.3 plasmids, respectively. The three independent ectromelia I5L clones were then sequenced using an Applied Biosystems 373 Sequencer.

2.5 Plasmids

2.5.1 EGFP fusion constructs for uninfected cells

I5L fragments destined for cloning into EGFP expression vectors were first cloned into pGEM-T (a summary of all fusion constructs is shown in Figure 10). The pGEM-T/I5L plasmid was constructed by generating the fragment for full-length I5L using PCR from purified VV65 DNA and using primer #1, GAATTCATGGTGGATGCTATAACC, containing an *EcoR* I restriction site, and primer #7, GGATCCTTAACTTTTCATTAATAG, containing a *BamH* I restriction site. All subsequent I5L constructs used this parental pGEM-T/I5L plasmid as a DNA template for PCR. pEGFP-C2/I5L was generated by liberating the I5L fragment from pGEM-T/I5L using *EcoR* I and *BamH* I restriction enzymes and ligating this fragment in to the EGFP-C2 vector (Clontech Laboratories Inc.). Fragments cloned into this vector will have EGFP fused to their N-terminus. The full-length I5L construct, pGEM-T/I5L(NS), lacking a stop codon, was generated using primer #1, GAATTCATGGTGGATGCTATAACC, containing an *EcoR* I restriction site, and primer #2, GGATCCACTTTTCATTAATAGGG, containing a *BamH* I restriction site, followed by ligation into the pGEM-T vector. The "NS" in this, and subsequent constructs, is used to denote the lack of a stop codon; necessary for fusion of EGFP to the C-terminus of inserts in the pEGFP-N3 vector. pEGFP-N3/I5L was constructed by liberating the I5L(NS) fragment from pGEM-T/I5L(NS) using *EcoR* I and *BamH* I restriction enzymes, and ligating this fragment into the pEGFP-N3 vector (Clontech Laboratories Inc.). A C-terminal truncation expressing only residues 1-69, pGEM-

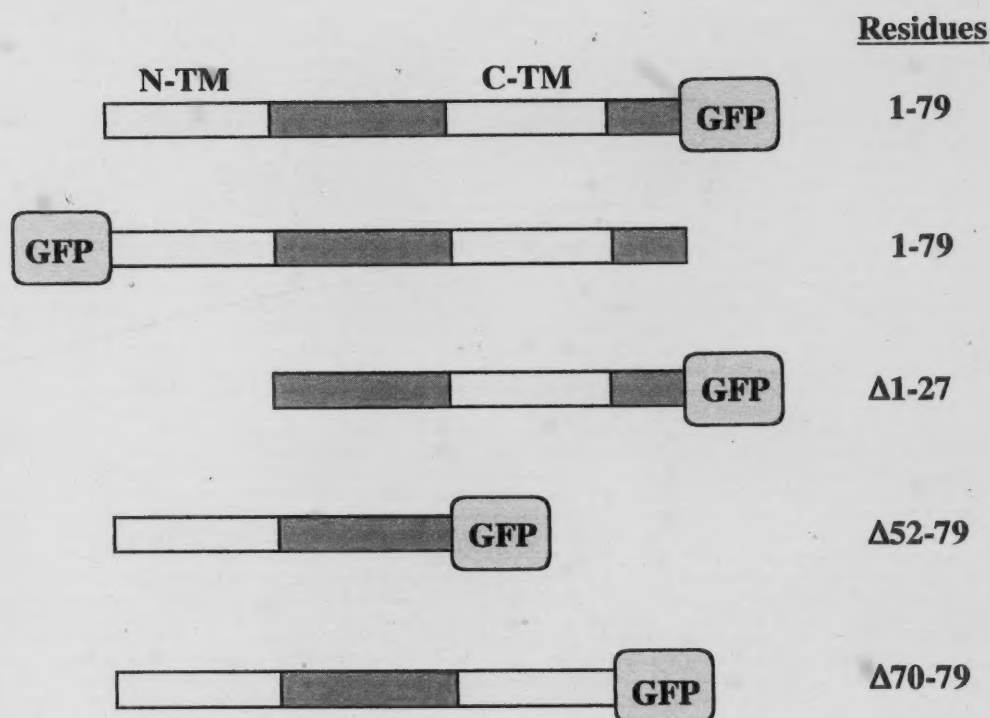


Figure 10. Summary of I5L-GFP Constructs. A complete listing of EGFP constructs for I5L is shown above. The N-terminal and C-terminal transmembrane domains (yellow) are labelled N-TM and C-TM, respectively. The intervening polar region and short polar tail (blue) are also represented. Enhanced green fluorescent protein (green) was fused to either the N- or C-terminus of each construct. Residue numbers describing each construct are provided as well as the primers used to generate the construct. (Primer numbers were obtained from Table 5). The constructs containing residues 1-79 represent full-length constructs.

T/I5L(Δ 70-79)NS, was generated using primer #1, GAATTCATGGTGGATGCTATAACC, containing an *EcoR* I restriction site, and primer #5, GAATTCATGGTGGATGCTATAACC, containing a *BamH* I restriction site and lacking a stop codon. pEGFP-N3/I5L(Δ 70-79) was generated by liberating the I5L(Δ 70-79)NS fragment from pGEM-T/I5L(1-70)NS using *EcoR* I and *BamH* I restriction enzymes, and ligating this fragment into the pEGFP-N3 vector. A C-terminal truncation, expressing only residues 1-51, pGEM-T/I5L(Δ 52-79)NS was generated using primer #1, GAATTCATGGTGGATGCTATAACC, containing an *EcoR* I restriction site, and primer #3, GGATCCAATCGTTACGGCTCGATT, containing a *BamH* I restriction site and lacking a stop codon. pEGFP-N3/I5L(Δ 52-79) was generated by liberating the I5L(Δ 52-79)NS fragment from pGEM-T/I5L(Δ 52-79)NS using *EcoR* I and *BamH* I restriction enzymes, and ligating this fragment into the pEGFP-N3 vector. An N-terminal truncation, pGEM-T/I5L(Δ 1-27)NS, expressing only residues 28-79 and lacking a stop codon, was generated using primer #4, GAATTCATGATAGTCAAGGAGTTA, containing an *EcoR* I restriction site, and primer #2, GGATCCACTTTTCATTAATAGGG, containing a *BamH* I restriction site. pEGFP-N3/I5L(Δ 1-27) was generated by liberating the I5L(Δ 1-27)NS fragment from pGEM-T/I5L(Δ 1-27)NS using *EcoR* I and *BamH* I restriction enzymes, and ligating this fragment into the pEGFP-N3 vector.

2.5.2 EGFP fusion constructs for expression during infection

To achieve expression of GFP and I5L-GFP fusion constructs during infection, the appropriate genes were placed under the control of a poxvirus early/late promoter in the pSC66 poxviral expression vector (kindly provided by Dr. Deborah Burshtyn, University of Alberta). pSC66/GFP was generated by liberating the GFP gene from the pEGFP-N3 vector using *Sal* I and *Not* I restriction enzymes, followed by ligation of the

liberated fragment into pSC66. Full-length I5L lacking a stop codon was generated by PCR using forward primer GTCGACACCATGGTGGATGCTATAACC, containing a *Sal* I restriction site and reverse primer GGATCCACTTTTCATTAATAGGG, containing a *Bam*H I restriction site, followed by ligation of this fragment into the TA cloning vector, pGEM-T, to yield pGEM-T/I5L-SB. To generate pEGFP-N3/I5L-SB, I5L was liberated from pGEM-T/I5L-SB using *Sal* I and *Bam*H I restriction enzymes and ligated into the pEGFP-N3 vector. To generate pSC66/I5L-EGFP, the I5L-EGFP fragment was liberated from pEGFP-N3/I5L-SB using *Sal* I and *Not* I restriction enzymes and ligated into the pSC66 vector, placing I5L-GFP under the control of a poxviral early/late promoter. To construct a C-terminally truncated I5L-GFP fusion, a fragment of I5L corresponding to residues 1-51 was generated by PCR using forward primer GTCGACACCATGGTGGATGCTATAACC, containing a *Sal* I restriction site and reverse primer GAATTCATGATAGTCAAGGAGTTA, containing a *Bam*H I restriction site. This PCR fragment was cloned into the TA cloning vector, pGEM-T, to yield pGEM-T/I5L(Δ 52-79)SB. The I5L(Δ 52-79) fragment was liberated from pGEM-T/I5L(Δ 52-79)SB using *Sal* I and *Bam*H I restriction enzymes and subcloned into the pEGFP-N3 vector to yield pEGFP-N3/I5L(Δ 52-79). The I5L(Δ 52-79)GFP fusion fragment was liberated from pEGFP-N3/I5L(Δ 52-79) using *Sal* I and *Not* I restriction enzymes and subcloned into the pSC66 vector to yield pSC66/I5L(Δ 52-79)GFP, thus placing the C-terminally truncated I5L-GFP fusion under the control of a poxviral early/late promoter. To construct an N-terminally truncated I5L-GFP fusion, a fragment of I5L corresponding to residues 28-79 was generated by PCR using forward primer GTCGACACCATGATAGTCAAGGAGTTA, containing a *Sal* I restriction site and reverse primer GGATCCACTTTTCATTAATAGGG, containing a *Bam*H I restriction site. This PCR fragment was cloned into the TA cloning vector, pGEM-T, to yield pGEM-T/I5L(Δ 1-27)SB. The I5L(Δ 1-27) fragment was liberated from pGEM-T/I5L(Δ 1-

27)SB using *Sal* I and *Bam*H I restriction enzymes and subcloned into the pEGFP-N3 vector to yield pEGFP-N3/I5L(Δ 1-27). The I5L(Δ 1-27)GFP fusion fragment was liberated from pEGFP-N3/I5L(Δ 1-27) using *Sal* I and *Not* I restriction enzymes and subcloned into the pSC66 vector to yield pSC66/I5L(Δ 1-27)GFP, thus placing the N-terminally truncated I5L-GFP fusion under the control of a poxviral early/late promoter.

2.5.3 Generation of *in vitro* translation expression constructs

DNA fragments destined for *in vitro* transcription and translation were generated by PCR. Full-length I5L was amplified using forward primer #11, GAATTCACCATGGTGGATGCTATAACC, possessing *Eco*R I and *Nco* I restriction sites, and primer #7, GGATCCTTAACTTTTCATTAATAG, containing a *Bam*H I restriction site. The amplified product was cloned into the TA-cloning vector, pGEM-T, to generate pGEM-T/I5L. A single adenine nucleotide was added immediately upstream of the *Nco* I site in the forward primer to complete the full Kozak's consensus sequence (KCS), ACCATGG. A C-terminally truncated version of I5L, I5L(Δ 52-79), was amplified using primer #11, GAATTCACCATGGTGGATGCTATAACC, possessing *Eco*R I and *Nco* I restriction sites, and primer #13, GGATCCTTAAATCGTTACGGCTAGATT, possessing a *Bam*H I restriction site, followed by ligation of the fragment into pGEM-T to yield pGEM-T/I5L(Δ 52-79). An N-terminally truncated version of I5L, I5L(Δ 1-27), was amplified using primer #11, GAATTCACCATGGTGGATGCTATAACC, possessing *Eco*R I and *Nco* I restriction sites, and primer #12, GAATTCACCATGGTAGTCAAGGAGTTA, possessing a *Bam*H I restriction site, followed by ligation of this fragment into the pGEM-T to yield pGEM-T/I5L(Δ 1-27). A full-length B6R fragment was amplified by PCR using primer #31, GAATTCACCATGGCCATGTCTTCTTCAGTGGAT, possessing *Eco*R I and *Nco* I sites, and primer #32, GGATCCTTATTTATACAACTAACTAG, possessing a *Bam*H I

restriction site. The resulting PCR fragment was ligated into the pGEM-T vector to yield pGEM-T/B6R. All pGEM-T constructs were verified by sequencing. pGEM-T constructs were digested using *Nco* I and *Bam*HI restriction enzymes to liberate inserts which were then subcloned into the pSPUTK *in vitro* translation vector (Falcone and Andrews, 1991) to generate the pSPUTK/I5L, pSPUTK/I5L(Δ 52-79), pSPUTK/I5L(Δ 1-27), and pSPUTK/B6R plasmids.

2.6 Transfections for Confocal Microscopy

HeLa or BHK cells were seeded on coverslips at densities of $2-3 \times 10^5$ cells/coverslip. Cells were transiently transfected with the desired I5L-GFP fusion. For each coverslip, 2 μ L of Lipofectamine 2000 (Invitrogen Life Technologies Inc.) was added to 50 μ L OptiMEM media (Invitrogen Life Technologies Inc.) to comprise "Solution A" which was incubated for 5 min at room temperature. "Solution B" contained 2 μ g of the appropriate I5L-EGFP plasmid in 50 μ L OptiMEM media. Solutions A and B were mixed together and incubated for 15 min at room temperature. Coverslips were washed once with warmed OptiMEM and the SolutionA/SolutionB transfection mixture was added along with 500 μ L OptiMEM. Transfections were incubated for 3 hours at 37°C, 5% CO₂ at which point 1 mL DMEM containing 15% FBS was added. Transfections were incubated at 37°C, 5% CO₂ for a further 4 h or 12 h for BHK and HeLa cells, respectively. To assess changes in localization of pre-expressed I5L upon infection, transfections were incubated for 6 hours at 37°C prior to infection with vaccinia virus strain Copenhagen at an MOI=5. Infections were incubated 4 hours at 37°C prior to fixation.

2.7 Preparation of Fixed Cells for Immunofluorescence Microscopy

Transfected cells were prepared for fixed-cell immunofluorescence confocal microscopy as follows. Cells were washed once using phosphate buffered saline (PBS) containing 1% FBS and then fixed for 10 minutes at room temperature with 2% paraformaldehyde (Sigma Chemical Co.) in PBS. After washing twice with PBS/1% FBS, cells were permeabilized with 0.2% NP-40 (Sigma Chemical Co.) for 5 minutes at room temperature followed by three more washes with PBS/1% FBS. Cells were then treated with primary antibodies by placing coverslips facedown on parafilm with 100 μ L of antibody solution. Primary antibodies used included an α -mannosidase II polyclonal antibody (a gift from Dr. T. Hobman, University of Alberta, Edmonton, Canada.) used at a 1:1000 dilution, an α -I7L polyclonal antibody and an α -G1L polyclonal antibody, both used at 1:500 dilution (both were kindly provided by Dr. D. Hruby, Oregon State University, Corvallis, OR, USA). Primary antibody incubation was performed for 16 h at room temperature. After incubation, cells were washed three times with PBS/1% FBS and treated with AlexaFluor546-conjugated goat α -rabbit IgG secondary antibodies (Molecular Probes Inc.) at a 1:500 dilution for 1 h at room temperature followed by three washes with PBS/1% FBS.

Specimens were stained with the DNA stain, Hoescht 33342 (Molecular Probes Inc.), at 500 ng/mL for 10 minutes at room temperature followed by three washes with PBS/1% FBS. GolgiTracker (Molecular Probes Inc.) was prepared by dissolution in minimal volume of anhydrous ethanol followed by dilution in Hank's Balanced Salt Solution (Invitrogen Life Technologies) to 5 μ M. GolgiTracker staining was achieved by incubating cells with 5 μ M GolgiTracker for 5 min at 4°C, followed by three successive washes with warmed OptiMEM media and incubation for 30 min at 37°C. ER Tracker (Molecular Probes Inc.) staining was achieved by treating cells with 0.5 mM ER Tracker for 30 min at 37°C, followed by three successive washes with warmed OptiMEM media. Coverslips were mounted onto microscope slides with an antifade reagent, *n*-propyl

gallate (Sigma), at 20 mg/mL in 1:1 PBS/glycerol. Confocal microscopy images were viewed using a Zeiss Axiovert 100M microscope equipped with 40X and 63X oil immersion Plan-Apochromat objective lenses, and a digital image capturer.

2.8 *In Vitro* Transcription and Translation

In vitro translation reactions were carried out using a coupled *in vitro* transcription/translation kit (Promega Corporation). As per the manufacturer's protocol, a 50 μ L reaction contained 50% rabbit reticulocyte lysate mixture, 20 μ M amino acid mixture (minus methionine), and 2.5% TNT reaction buffer. Components not included with the kit were 40 U RNasin (Promega), 10 μ Ci [^{35} S]methionine (Perkin-Elmer Life Sciences, Boston, MS, USA) at 1000 Ci/mmol, and 1 μ g of the appropriate pSPUTK DNA template. Reactions were incubated for 90 min at 30°C and were terminated by the addition of sodium dodecyl sulphate (SDS) sample buffer, followed by boiling for 10 min, at which point samples were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Gels were dried and exposed to Kodak BioMax MS film (Eastman Kodak Company), using a Kodak BioMax LE Intensifying Screen.

Insertion of the various constructs into microsomes was analyzed in both co- and post-translational assays. For co-translational insertion, 5 μ L of 100 A280/mL canine pancreatic microsomes (courtesy of Dr. David Andrews, McMaster University, Hamilton, Ontario, Canada) was added to reactions at the beginning of the coupled reaction. For post-translational insertion, 200 μ g/mL cycloheximide (Sigma) was added following the 90 min transcription/translation incubation. 5 μ L of 100 A280/mL canine pancreatic microsomes were then added followed by incubation at 30°C for 30 min. Reactions were centrifuged at 20,000 $\times$ g for 20 min at 4°C to separate the membranous material from the supernatant. The pellet was washed by with 200 μ L of PBS followed by centrifugation at 20,000 $\times$ g for 20 min at 4°C. Washed pellets were resuspended in 50 μ L PBS, layered on

top of a 100 μ L cushion of 0.5 M sucrose in 1 mM Tris pH 8.0, and centrifuged at 20,000 $\times$ g for 20 min at 4°C. The resulting pellet was resuspended in 50 μ L of PBS. SDS sample buffer was added to both supernatant and pellet fractions which were then boiled 10 min prior to analysis as described by SDS-PAGE. To verify membrane integration, pellets were prepared as described above, but washed with 200 μ L of 0.5 M Na₂CO₃ (pH 11.5) to disrupt non-specific membrane binding.

To differentiate between aggregated protein and bona fide membrane/protein interactions, vesicle flotation analysis was used (Millman and Andrews, 1999). Upon washing of membrane pellets with PBS, a 3-layered sucrose gradient was constructed containing 35 mM Tris acetate (Sigma), 190 mM potassium glutamate (Sigma), 30 mM ammonium acetate (Sigma) and 12 mM magnesium acetate (Sigma). The pellet was resuspended in 50 μ L of PBS and mixed with 100 μ L of 2.4 M sucrose, yielding a 1.6 M sucrose mixture which formed the bottom layer of the gradient. A 50 μ L layer of 1.25 M sucrose, and a 50 μ L layer of 0.25 M sucrose were then gently layered onto the 1.6 M sucrose/reaction mixture. Gradients were poured in Beckman Ultraclear 8mm x 49mm tubes and centrifuged at 100,000 $\times$ g for 90 min at 4°C in a Beckman-Coulter Optima L-90K Ultracentrifuge using a Sorvall 50.4Ti rotor. Following centrifugation, three 80 μ L fractions were removed from top to bottom for each sample. A fourth fraction for each sample was obtained by washing out the tube with 80 μ L of PBS to resuspend any material that may have pelleted. The samples were then boiled for 10 min in SDS sample buffer and analyzed by SDS-PAGE.

Membrane topology was explored by digesting inserted proteins with trypsin or proteinase K. Following vesicle flotation analysis and separation of the gradient into 80 μ L fractions, either 2.5 μ g/mL of trypsin (Grade III, Sigma Chemical Co.) or 2.5 μ g/mL of proteinase K were added to the appropriate samples and incubated at 30°C for 1 h.

SDS sample buffer was added to the samples which were then boiled for 10 minutes and analyzed by SDS-PAGE.

2

Results

3.1 The I5L orthologue from ectromelia virus is highly conserved

Ectromelia virus (EV), a member of the *Orthopoxviridae* genus, is a highly pathogenic poxvirus of mice that often serves as an animal model for studying the *in vivo* functions of poxviral proteins. Since the genome sequence of EV had not been completed, we successfully cloned and sequenced an I5L orthologue from EV. When this sequence was included in an alignment with previously sequenced poxviral orthologues (Figure 9), as expected it clearly fell amongst the other orthopoxviruses as opposed to members of other poxviral genera in the bottom half of the alignment. When compared with vaccinia virus (VV), the EV orthologue contained only a single amino acid substitution -- a conservative alanine-to-threonine change at residue 26. This recapitulates the extremely high conservation of I5L seen amongst the orthopoxviruses.

3.2 I5L localizes to a perinuclear region in infected cells

One of the hypotheses of my thesis was that I5L is a tail-anchored protein. With the presence of a putative transmembrane segment at each end of I5L, a reasonable prediction was that I5L targets to a membranous subcellular structure. To determine if these hypotheses were correct, we fused a jellyfish green fluorescent protein (GFP) tag onto I5L in order to visualize its subcellular localization. In order to assure that transfected I5L would be expressed at high levels during infection, we placed the I5L-GFP fusion under the control of a synthetic poxviral early/late promoter. An added benefit of such a system is that uninfected cells should not express the fusion even if they are transfected.

When baby hamster kidney (BHK) cells were transfected with the GFP control plasmid pSC66/GFP and infected with wildtype vaccinia virus, infected cells displayed

the diffuse GFP staining pattern expected for a soluble protein (Figure 11, panels A-C). When BHK cells were transfected with pSC66/I5L-GFP, infected cells displayed a compact perinuclear staining pattern (Figure 11, panels D-F). The GFP staining pattern of I5L-GFP did not resemble either the ER staining profile of the tail-anchored vaccinia protein B6R (Figure 5), or the mitochondrial staining pattern of the tail-anchored protein F1L (Figure 6). The pattern instead resembled the Golgi compartment, to which several vaccinia proteins are known to localize during infection (Lorenzo *et al.*, 2000).

3.3 I5L does not localize to the Golgi compartment in infected cells

In order to determine if the structure to which I5L localized was the Golgi compartment, we stained infected/transfected cells with a polyclonal antibody specific for the cellular protein, mannosidase II, which localizes specifically to the *cis*-medial Golgi compartment (Novikoff *et al.*, 1983). Upon staining for mannosidase II, mock-infected cells displayed a compact perinuclear, Golgi compartment (Figure 12, panel A). In contrast, infected cells often displayed a fragmented Golgi compartment (Figure 12, panel C). When I5L-GFP was transfected into BHK cells, the resulting perinuclear GFP targeting did not colocalize to any noticeable extent with the mannosidase II antibody (Figure 12, panels D and E). These observations indicated that I5L-GFP does not localize to the Golgi compartment during infection.

3.4 I5L localizes to the viral factory in infected cells

With the Golgi compartment ruled out, another strong candidate for the site of I5L localization was the viral factory. The viral factory is a structure which forms in the cytoplasm of infected cells and represents the site of virion assembly (Gaylord and Melnick, 1953). It is enriched in viral membranes, viral genomic DNA and a wide assortment of virion proteins, both structural and enzymatic (Joklik and Becker, 1964;

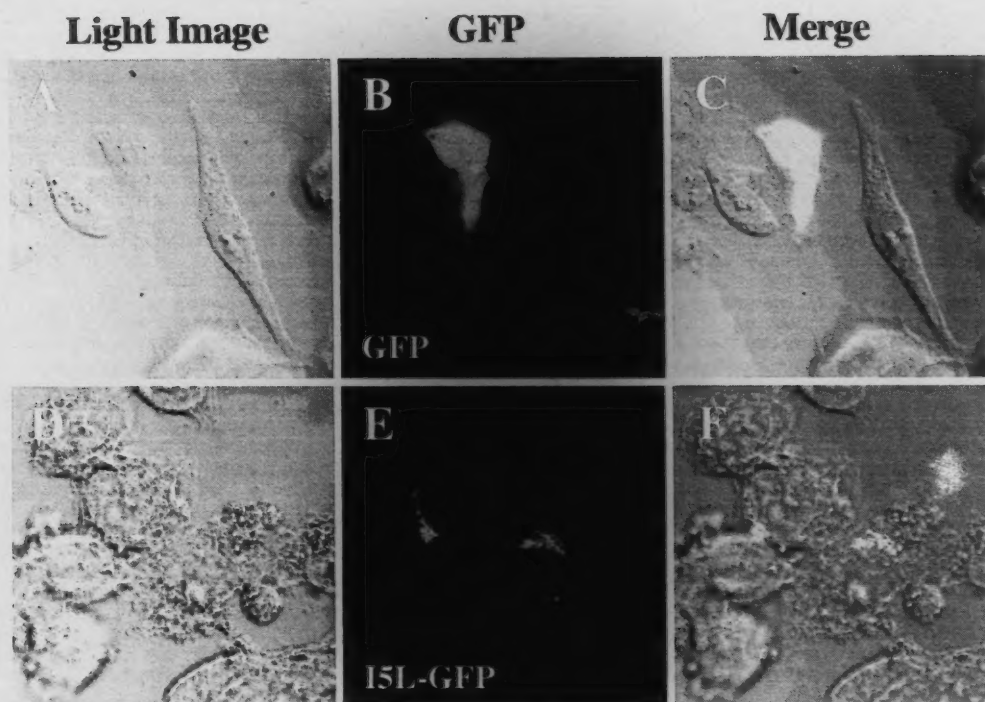


Figure 11. I5L localizes to a perinuclear region in infected cells

Fixed-cell confocal microscopy images were taken of BHK cells which were transiently transfected with either a GFP control plasmid (panels A-C) or a full-length I5L-GFP fusion (panels D-F) under the control of a poxviral promoter. Concurrent with transfection, cells were infected for 9h with wildtype vaccinia virus. A light microscopy image (panels A and D) and GFP emission (panels B and E) are shown. A merged image are is also shown (panels C and F).

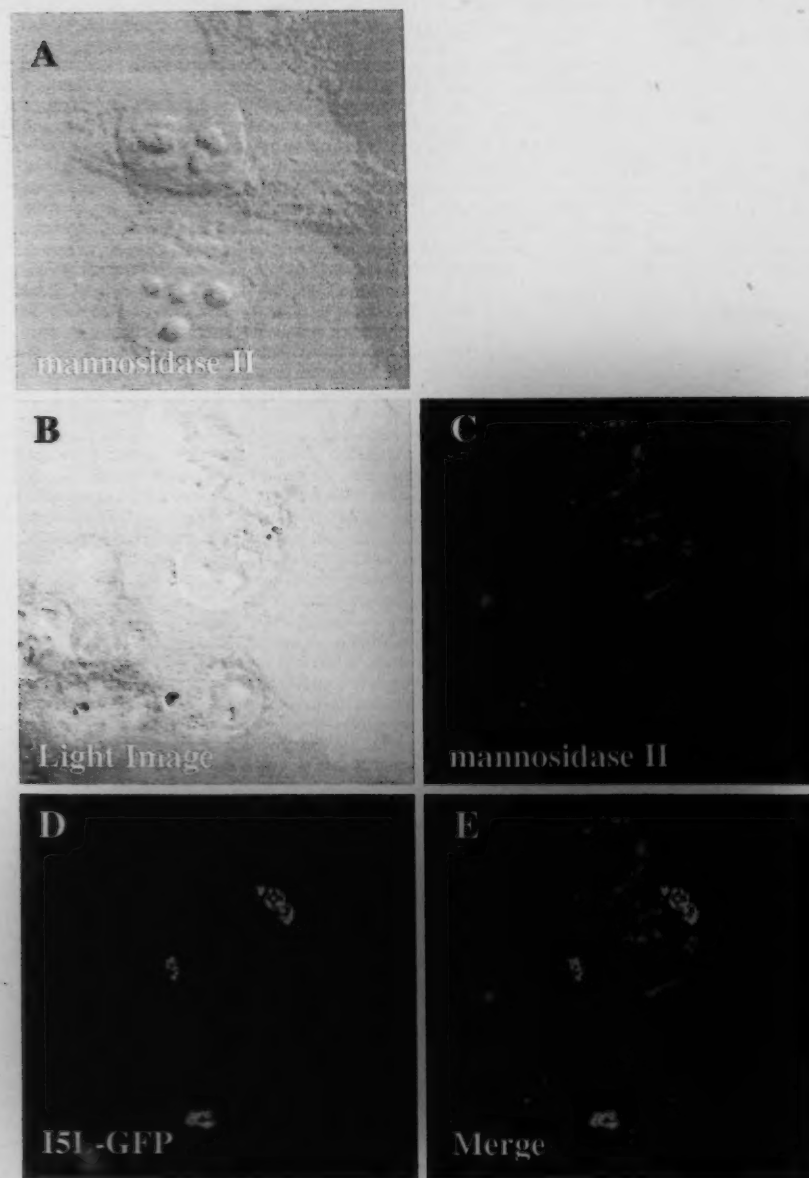


Figure 12. I5L does not localize to the Golgi compartment during infection

Fixed-cell confocal microscopy images were taken of BHK cells which were transiently transfected with a full-length I5L-GFP fusion under the control of a poxviral promoter. Concurrent with transfection, cells were either mock infected (panel A) or infected with wildtype vaccinia virus (panels B-E) for 9h. Several different emissions were visualized: a light microscopy image (panel B), the polyclonal mannosidase II antibody (panel A and C), GFP emission (panel D), and a merged image of GFP and α -mannosidase II emissions (panel E). An uninfected cell stained with α -mannosidase II is also shown (panel A).

Dales and Mosbach, 1968; Tsuruhara, 1971). A simple way to identify viral factories in infected cells is to stain cells with the fluorescent DNA stain Hoescht 33342 (Welsch *et al.*, 2003). This DNA-specific stain will stain all DNA in the cell -- both host DNA in the nucleus and viral genomic DNA in factories. When mock-infected cells were stained with Hoescht 33342 and visualized by confocal microscopy, only the nuclei were visible (Figure 13, panel A). There was no evidence of cytoplasmic DNA-containing viral factories. When infected cells were stained with Hoescht 33342, both nuclear DNA and cytoplasmic DNA was evident (Figure 13, panel C). Cytoplasmic viral factories was organized into clumped, compact structures which were usually, but not always perinuclear. They ranged in size from only barely visible to almost as large as the nucleus. Infected cells usually contained 1 or 2 distinct factories, but sometimes up to 5 or 6. When cells were transfected with pSC66/I5L-GFP, infected with wildtype vaccinia, and stained with Hoescht 33342, cytoplasmic I5L-GFP aggregates colocalized nicely with the DNA stain (Figure 13, panels D and E) suggesting that I5L localized to viral factories in infected cells.

3.5 I5L co-localizes with viral factory markers during infection

We wanted to solidify our assertion that these structures were in fact viral factories by utilizing antibodies specific for the factory. We chose the viral factory proteins I7L and G1L. I7L is a vaccinia core proteinase that is packaged into virions (Byrd *et al.*, 2002). It would presumably be found in viral factories. G1L is another core proteinase and is also expected to localize to viral factories (Whitehead and Hruby, 1994; Byrd *et al.*, 2002). I7L did not show significant labelling of mock infected cells (Figure 14, panel A). Upon infection, I7L antibodies stained the DNA-rich cytoplasmic factories (Figure 14, panels B and D). Upon transfection of infected cells with pSC66/I5L-GFP,

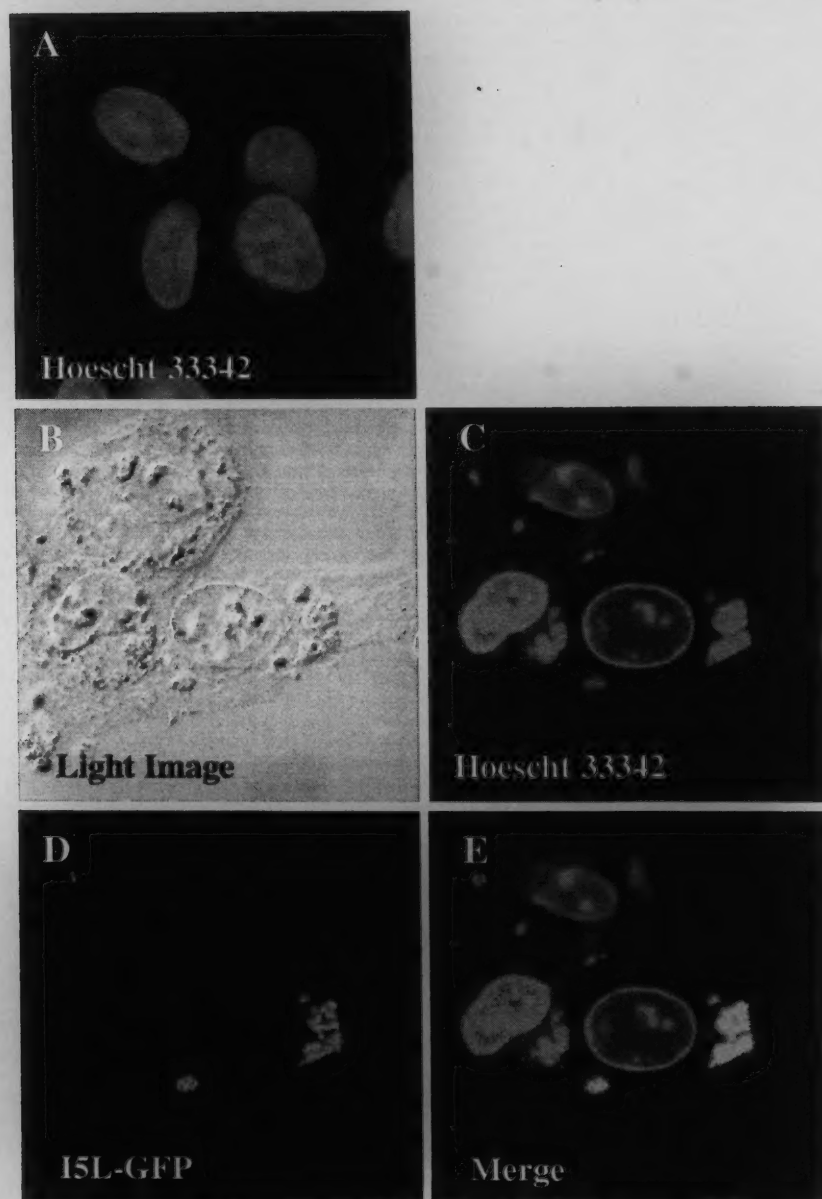


Figure 13. I5L localizes to a DNA-rich cytoplasmic structure in infected cells

Fixed-cell confocal microscopy images were taken of BHK cells which were transiently transfected with a full-length I5L-GFP fusion under the control of a poxviral promoter. Concurrent with transfection, cells were infected with wildtype vaccinia virus (panels A-E) for 9h at MOI=3. Several different emissions were visualized: a light microscopy image (panel A), the nonspecific DNA dye, Hoescht 33342 (panel B), GFP emission (panel C), and a merged image of Hoescht and GFP emissions (panel D). Hoescht staining of mock-infected cells are also shown (panel E).

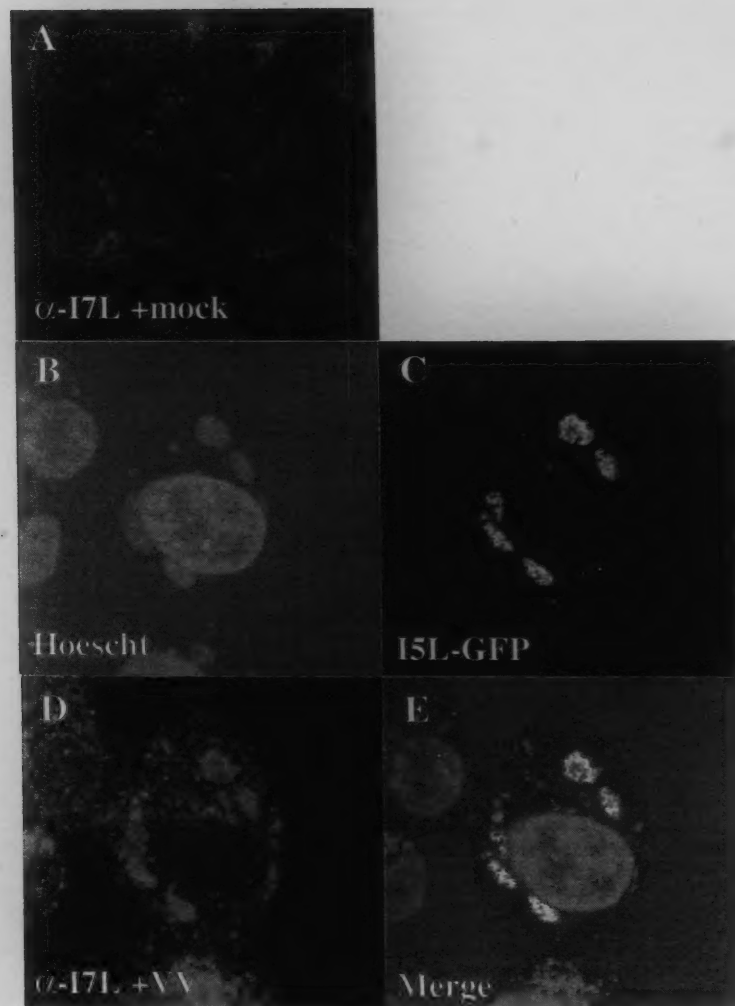


Figure 14. I5L colocalizes with the viral factory marker, I7L, during infection. Fixed-cell confocal microscopy images were taken of BHK cells which were transiently transfected with a full-length I5L-GFP fusion, I5L-GFP. Concurrent with transfection, cells were mock infected (panel A) or infected with vaccinia virus strain Copenhagen (panels B-E) for 9h at an MOI of 3. Several different emissions were visualized: Hoescht 33342 (panel B), GFP emission (panel C), and an I7L polyclonal antibody (panels A and D). A merged image of panels B-D is also shown (panel E).

the I5L-GFP fusion protein co-localized with I7L in the viral factories (Figure 14, panels C and E).

G1L did not show significant labelling of mock infected cells (Figure 15, panel A). Upon infection, G1L antibodies stained the DNA-rich cytoplasmic factories (Figure 15, panels B and D). Upon transfection of infected cells with pSC66/I5L-GFP, the I5L-GFP fusion protein co-localized with G1L in the viral factories (Figure 15, panels C and E). Co-localization of I5L-GFP with G1L was much cleaner than co-localization with I7L since the I7L polyclonal antibody was much more dispersed throughout the cell. It is not clear whether this reflects the actual localization pattern for I7L during infection, or whether the I7L antibody is just less specific than the G1L antibody.

It is interesting to note that while I5L-GFP and G1L both localized to viral factories and displayed a punctate staining profile, the punctate patterns were not superimposable and were in fact mutually exclusive (Figure 16, panels B-F) suggesting that I5L and G1L localize to distinct subdomains of the viral factory. No such colocalization pattern was evident between I5L-GFP and I7L.

3.6 The N-terminus of I5L is necessary for factory targeting, while the C-terminus is not

At the outset, one of the goals of these studies was to determine which regions of I5L were important for subcellular targeting, if such targeting was observed. We have already shown that I5L targets to the viral factory during infection. In order to identify which regions of I5L were important for this targeting, we created I5L truncations fused to GFP and visualized their localization using confocal microscopy as before.

The C-terminal truncation construct, I5L(Δ 52-79)GFP expresses I5L residues 1-51, with GFP fused to the C-terminus, and is thus lacking the tail-anchoring motif. BHK cells were infected with wildtype vaccinia virus and transfected with I5L(Δ 52-79)GFP.

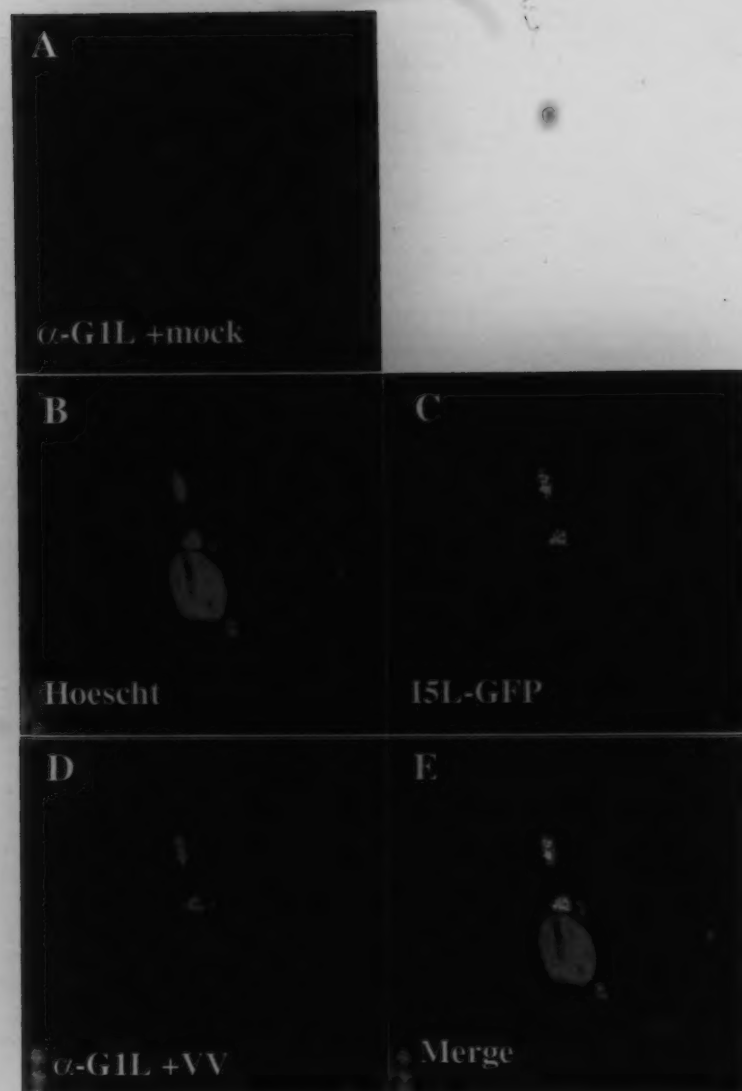


Figure 15. I5L-GFP colocalizes with the viral factory marker, G1L, during infection. Fixed-cell confocal microscopy images were taken of BHK cells which were transiently transfected with a full-length I5L-GFP fusion, I5L-GFP. Concurrent with transfection, cells were either mock infected (panel A) or infected with vaccinia virus strain Copenhagen (panels B-E) for 9h at an MOI of 3. Several different emissions were visualized: Hoescht 33342 (panel B), GFP emission (panel C), and an G1L polyclonal antibody (panel A and D). A merged image of panels B-D is also shown (panel E).

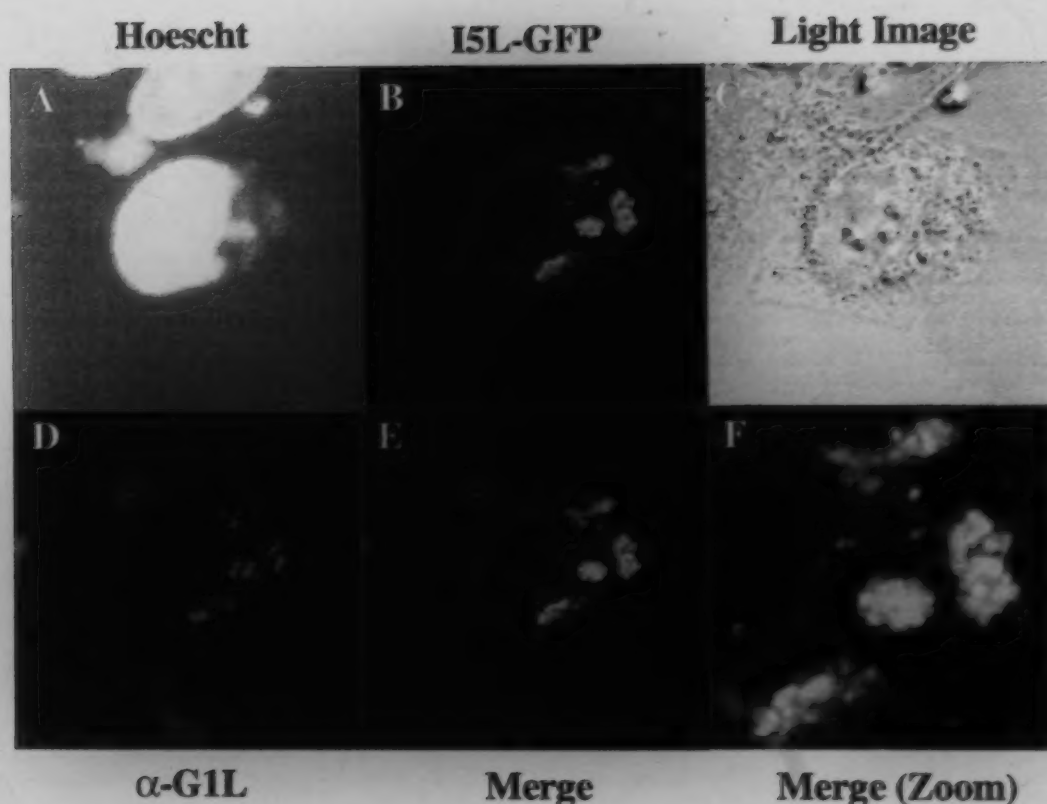


Figure 16. I5L and G1L localize to distinct sub-domains of the viral factory

Fixed-cell confocal microscopy images were taken of BHK cells which were transiently transfected with a full-length I5L-GFP fusion under the control of a poxviral promoter. Concurrent with transfection, cells were infected with vaccinia virus strain Copenhagen (Panels A-F) for 9h at an MOI of 3. Several separate emissions were visualized: Hoescht 33342 (panel A), a fluorescent GFP emission (panel B), a light microscopy image (panel C), and a G1L polyclonal antibody (panel D). A merged image of GFP and G1L emissions is shown (panel E), and is also magnified (panel F).

To our surprise, the fusion construct still localized to the viral factory as demonstrated by its colocalization with Hoescht 33342 and the viral factory marker and G1L (Figure 17, panels A-E). In fact, the localization pattern of truncated I5L-GFP was indistinguishable from full-length I5L using all available factory markers. The observation that a C-terminally truncated I5L can still localize to viral factories implies that the N-terminus of I5L must be interacting with a component of the viral factory. Whether this interaction involves membrane material in the factory, or is a result of protein-protein interactions with a cellular or viral protein is unknown.

I5L(Δ 52-79)GFP localizes to a distinct sub-domain of the viral factory from G1L (Figure 17, panel F) in an identical manner to full-length I5L (compare with Figure 16, panel F). This suggests that the region of I5L that distinguishes its factory labeling from that of G1L is found in the N-terminal half of the protein.

Since the N-terminus of I5L is sufficient to target the protein to the viral factory during infection, we were thus interested in determining where the C-terminal tail-anchoring motif of I5L would target the protein if the N-terminus was truncated. This would help us to determine if the C-terminus was actually a tail-anchoring domain. We constructed an I5L-GFP fusion, I5L(Δ 1-27)GFP, in which the N-terminal 27 amino acids were deleted. This construct thus lacks the putative N-terminal signal sequence. When infected BHK cells were transfected with this construct, the fusion protein no longer localized to the viral factory as shown by its lack of colocalization with viral factory markers Hoescht 33342 and G1L (Figure 18, panels A-E). I5L(Δ 1-27)GFP instead accumulated in a perinuclear structure adjacent to the nucleus, which is suggestive of Golgi compartment localization. To verify if this structure was the Golgi compartment, cells were transfected with I5L(Δ 1-27)GFP and stained with a mannosidase II polyclonal antibody. I5L(Δ 1-27)GFP was found to localize very specifically to the Golgi compartment as shown by its overlap with mannosidase II (Figure 19, panels A-E).

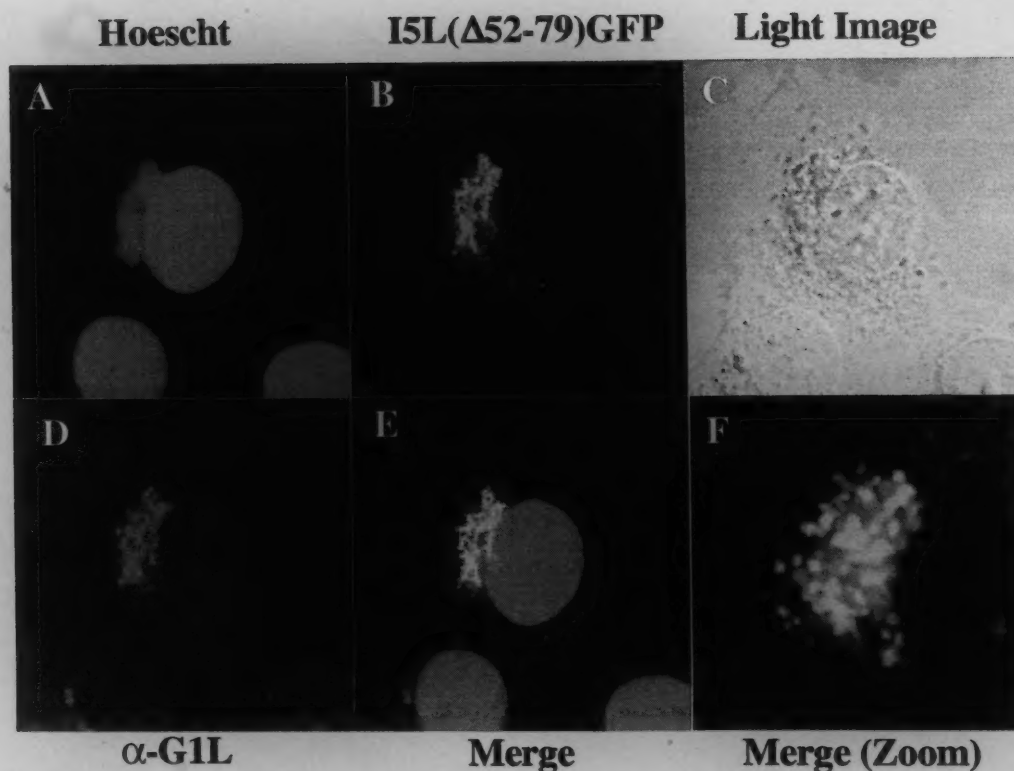


Figure 17. A C-terminally truncated I5L-GFP fusion protein colocalizes with G1L in infected cells

Fixed-cell confocal microscopy images were taken of BHK cells which were transiently transfected with a C-terminally truncated I5L-GFP fusion, I5L(Δ52-79)GFP. These fusions are under the control of a poxviral promoter and will therefore only be expressed in infected cells. Concurrent with transfection, cells were infected with vaccinia virus strain Copenhagen for 9h at an MOI of 3. Several different emissions were visualized: Hoescht 33342 (panel A), GFP emission (panel B), a light microscopy image (panel C), and a G1L polyclonal antibody (panel D). A merged image is also shown (panel E), and is magnified (panel F).

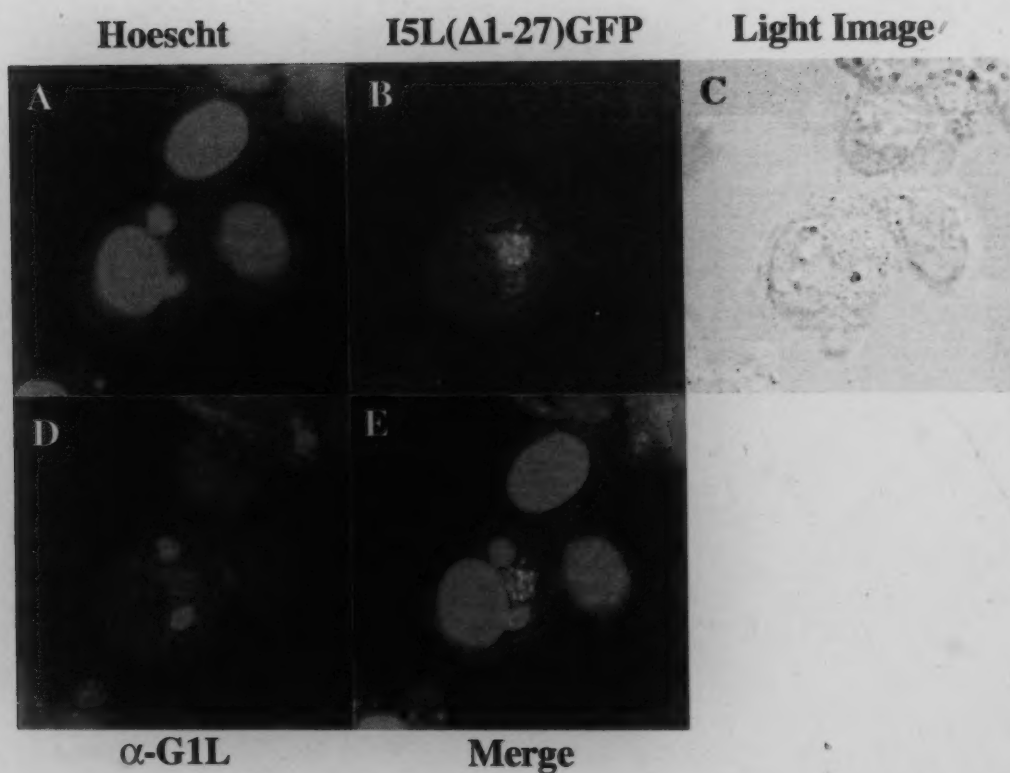


Figure 18. An N-terminally truncated I5L-GFP fusion protein does *not* colocalize with viral factory markers during infection

Fixed-cell confocal microscopy images were taken of BHK cells which were transiently transfected with an N-terminally truncated I5L-GFP fusion, I5L(Δ1-27)GFP. Concurrent with transfection, cells were infected with vaccinia virus strain Copenhagen for 9h at an MOI of 3. Several different emissions were visualized: Hoescht 33342 (panel A), GFP emission (panel B), a light microscopy image (panel C), and a G1L polyclonal antibody (panel D). A merged image is also shown (panel E).

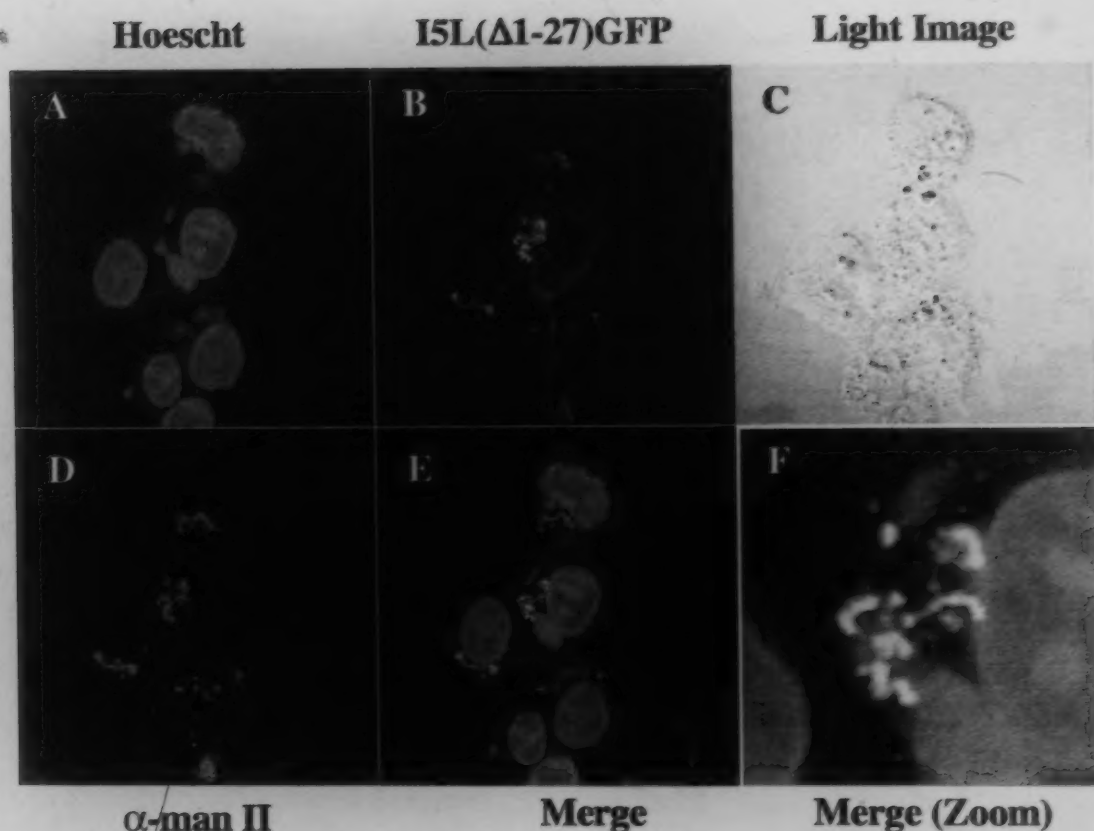


Figure 19. An N-terminally truncated I5L-GFP fusion protein localizes to the Golgi compartment during infection

Fixed-cell confocal microscopy images were taken of BHK cells which were transiently transfected with an N-terminally truncated I5L-GFP fusion, I5L(Δ 1-27)-GFP. Concurrent with transfection, cells were infected with vaccinia virus strain Copenhagen for 9h at an MOI of 3. Several different emissions were visualized: Hoescht 33342 (panel A), GFP emission (panel B), a light microscopy image (panel C), and a mannosidase II polyclonal antibody (panel D). A merged image is also shown (panel E), and is magnified (panel F).

We were also able to show that I5L which was targeted to the Golgi compartment in the absence of infection was unable to leave the Golgi for the factory upon subsequent infection (Figure 20, panels A-H). This was observed in cells transfected for 8 hours prior to infection with wild-type vaccinia virus.

3.7 I5L inserts into co-translationally into canine pancreatic microsomes in an *in vitro* assay

One of our initial hypotheses was that I5L was a tail-anchored protein since it possessed a tail-anchoring motif. One of the features of tail-anchored proteins is that they contain their targeting information in and/or near their hydrophobic C-termini (Borgese *et al.*, 2003). From our confocal studies with I5L-GFP fusions it became clear that the N-terminus of I5L played a critical role in targeting I5L to viral factories during infection. However, we could not ignore the observation that the hydrophobic C-terminus was able to mediate Golgi compartment localization if the N-terminus was missing. This suggested that both termini of I5L may play a role in targeting I5L during infection, perhaps by first targeting to the Golgi compartment in a process mediated by the C-terminus, and then localizing to the factory in a process mediated by the N-terminus.

The evidence thus far was suggestive with I5L being a membrane protein and that the C-terminal domain mediated membrane localization. This evidence includes (1) the reported isolation of I5L from virion membranes (Takahashi *et al.*, 1994), (2) the unusually high hydrophobicity of I5L (Figure 9), (3) the localization of full-length I5L to viral factories which are rich in virion membranes, and (4) the localization of an N-terminally truncated I5L to the Golgi compartment. Therefore, to determine if I5L was a *bona fide* membrane protein, we employed a commercially available coupled *in vitro* transcription and translation (TNT) assay. We made use of the very efficient *in vitro* TNT

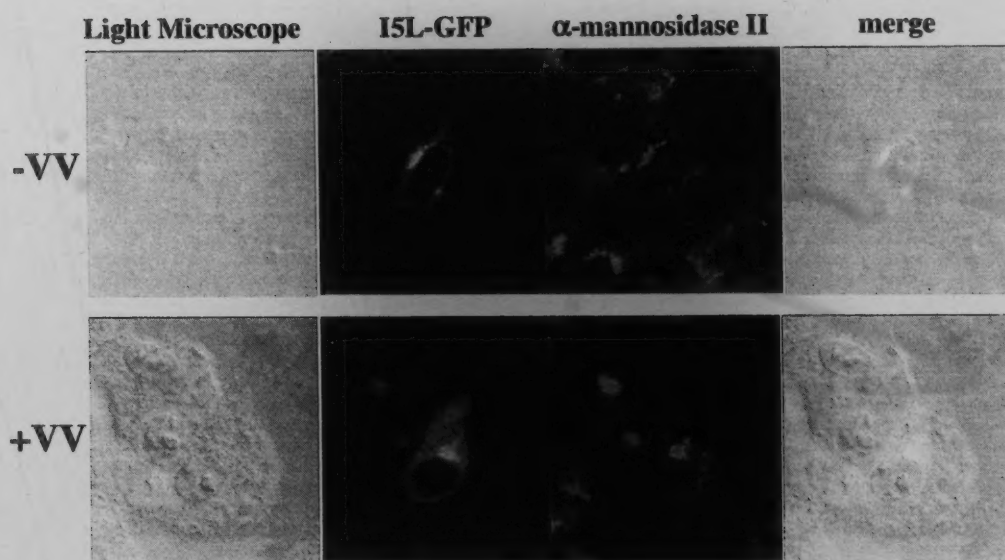


Figure 20. Vaccinia Infection Does Not Alter I5L-GFP Localization

BHK cells were transiently transfected with I5L-GFP. Cells were then either mock-infected or infected with VV strain WR for 5 hours at an MOI of 3. Cells were fixed and stained with a mannosidase II polyclonal antibody. Although infected cells displayed extensive cytopathic effects, I5L-GFP remained predominantly localized to the Golgi compartment as evidenced by its continued co-localization with mannosidase II.

vector, pSPUTK (Falcone and Andrews, 1991). Inserts cloned into the multiple cloning site of this vector are placed under the control of an SP6 promoter and the 5' untranslated leader of the *Xenopus laevis* β -globin gene. The β -globin 5' UTR is a strong promoter of translation initiation and greatly increases the efficiency of *in vitro* translation (Falcone and Andrews, 1991). Genes or gene fragments of interest were cloned into this vector such that the inserts were transcribed and translated in the presence of [^{35} S]methionine and visualized by autoradiography following separation by SDS-PAGE.

Although I5L is a small protein, only 79 amino acids, it contains 6 methionine residues. It should therefore be readily detectable by autoradiography. When full length I5L was expressed in this assay, a band was seen between the 7- and 17-kDa ladder markers (Figure 21, lane 1). This size is consistent with the predicted 9-kDa size of I5L. This band was not seen when empty vector was used as a TNT template (data not shown).

In order to determine if I5L was a transmembrane protein, membrane insertion assays were carried out using canine pancreatic microsomal membranes (CPMM). Canine pancreatic microsomes are essentially a vesicular fraction of ER-rich membranes obtained by subcellular fractionation of canine pancreatic cells (Scheele, 1983). They contain the complete repertoire of ER membrane proteins; most importantly those involved in co-translational protein translocation into the endoplasmic reticulum.

Insertion of tail-anchored proteins into membranes occurs post-translationally, whereas most other proteins, especially those possessing a signal sequence are inserted co-translationally. To assess co-translational insertion we translated I5L in the presence of CPMMs. Following completion of translation, reactions were separated into supernatant and microsomal membrane fractions. When I5L was translated in the absence of microsomes, all of the radiolabelled protein was found in the supernatant fraction as expected (Figure 21, lanes 1 and 2). When I5L was translated in the presence of

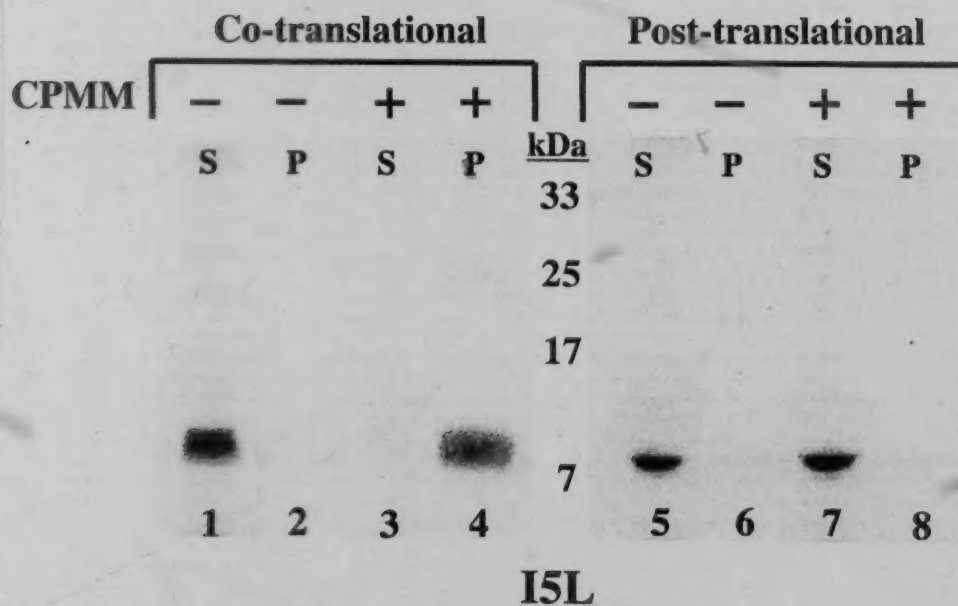


Figure 21. I5L inserts into membranes co-translationally, but not post-translationally. [^{35}S]-labelled full-length I5L was generated using a coupled *in vitro* transcription/translation assay. Canine pancreatic microsomal membranes (CPMM) were added during translation (lanes 3 and 4) or after the addition of cycloheximide (lanes 7 and 8) in order to facilitate co- or post-translational I5L insertion respectively. Microsomal membranes were separated from supernatants by centrifugation and both supernatant (S) and pellet (P) fractions were analyzed by SDS-PAGE.

microsomes virtually all of the radiolabelled protein was found in the pellet fraction (Figure 21, lanes 3 and 4). This is consistent with co-translational insertion of I5L into membranes. It is interesting to note that soluble and microsome-associated forms of I5L migrated at the same size following SDS-PAGE separation (compare lanes 1 and 4), indicating that signal peptide cleavage of the putative signal sequence did not occur. Such cleavage would be readily visible since almost a third of the protein would be removed. It must also be noted that this assay, on its own, cannot distinguish between co- and post-translational insertion. Since the TNT reaction is incubated for 90 minutes in the presence of microsomes, there is almost surely enough time for proteins to be translated, folded and post-translationally inserted into microsomes (Kim *et al.*, 1997). Observations from co-translational insertion assays must be combined with observations from post-translational insertion assays in order to make any conclusions as to the mechanism of I5L insertion.

Post-translational insertion assays were carried out by translating the protein of interest in the absence of microsomes. Following translation, the translation inhibitor cycloheximide was added to prevent further translation. Microsomes were then added to the reaction to allow for post-translational microsome insertion. When I5L was translated in this assay, virtually all translated protein remained in the supernatant fraction (Figure 21, lanes 5-8). I5L, therefore, did not display the membrane insertion pattern expected for tail-anchored proteins.

The vaccinia B6R protein was used as a positive control for post-translational insertion. Unpublished data from our laboratory has indicated that B6R behaves like a tail-anchored protein using GFP-tagged versions of B6R under confocal microscopy (refer to Figures 5 and 6). Additionally, sequence analysis of B6R identified a single transmembrane domain, located at the extreme C-terminus of the protein, which is a characteristic feature of tail-anchored proteins. When full-length B6R was expressed

from the pSPUTK vector in our TNT assay, a prominent band close to the expected 20-kD was seen (Figure 22, lane 1). When membranes were present during translation, most of the protein migrated into the pellet fraction (lanes 3 and 4). When membranes were added after translation and the addition of cycloheximide, most of the translated B6R still migrated into the pellet fraction (lanes 5 and 6). The post-translational assay allows only post-translational insertion to occur, while the co-translational assay likely permits both co- and post-translational insertion. In instances where both versions of the assay displayed similar supernatant/pellet distributions of radiolabelled protein, it was concluded that the membrane-associated portion was solely the result of post-translational insertion.

3.8 The C-terminus of I5L associates with microsomes *in vitro*, while the N-terminus does not.

Co- and post-translational insertion patterns for full length I5L, while confirming that I5L is a membrane protein, did not help to delineate the role(s) of the N- and C-terminal hydrophobic domains in membrane insertion. In order to assess the role(s) of these regions, two truncation constructs were analyzed, with the expectation that C-terminus, and possibly the N-terminus, would anchor into microsomal membranes. This prediction was based on our observations in infected cells in which both termini were able to achieve specific subcellular targeting to membranous sites.

The first construct, I5L(Δ 1-27), had the N-terminal 27 amino acids deleted; this region corresponded to the putative signal sequence. When this construct was analyzed in the *in vitro* TNT assays, approximately half of the radiolabelled protein was found in the membrane pellet in post-translational assays (Figure 23, lanes 1-4), with the other half remaining in the supernatant fraction. Tail-anchored proteins often display an incomplete movement of protein from the supernatant to the pellet fractions, as can be seen with B6R

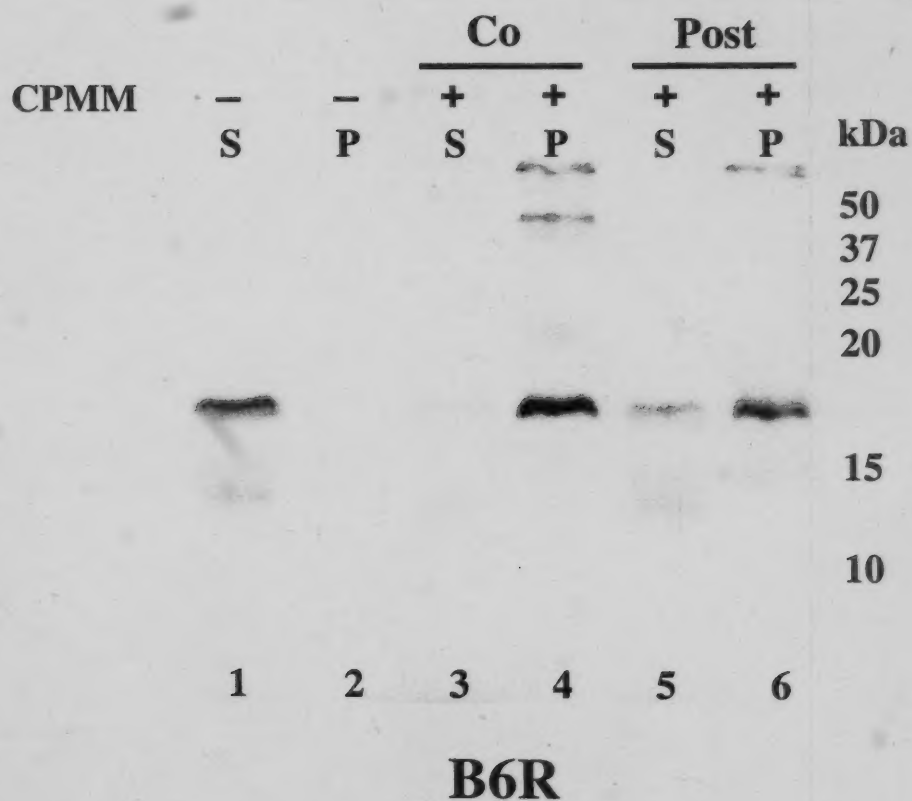


Figure 22. B6R inserts into membranes post-translationally
 $[^{35}\text{S}]$ -labelled proteins were generated using a coupled *in vitro* transcription/translation assay. Canine pancreatic microsomal membranes (CPMM) were either not added (lanes 1 and 2), added during translation (lanes 3 and 4), or after translation and the addition of cycloheximide (lanes 5 and 6). Microsomal membranes were separated from supernatants by centrifugation and both supernatant (S) and pellet (P) fractions were analyzed by SDS-PAGE.

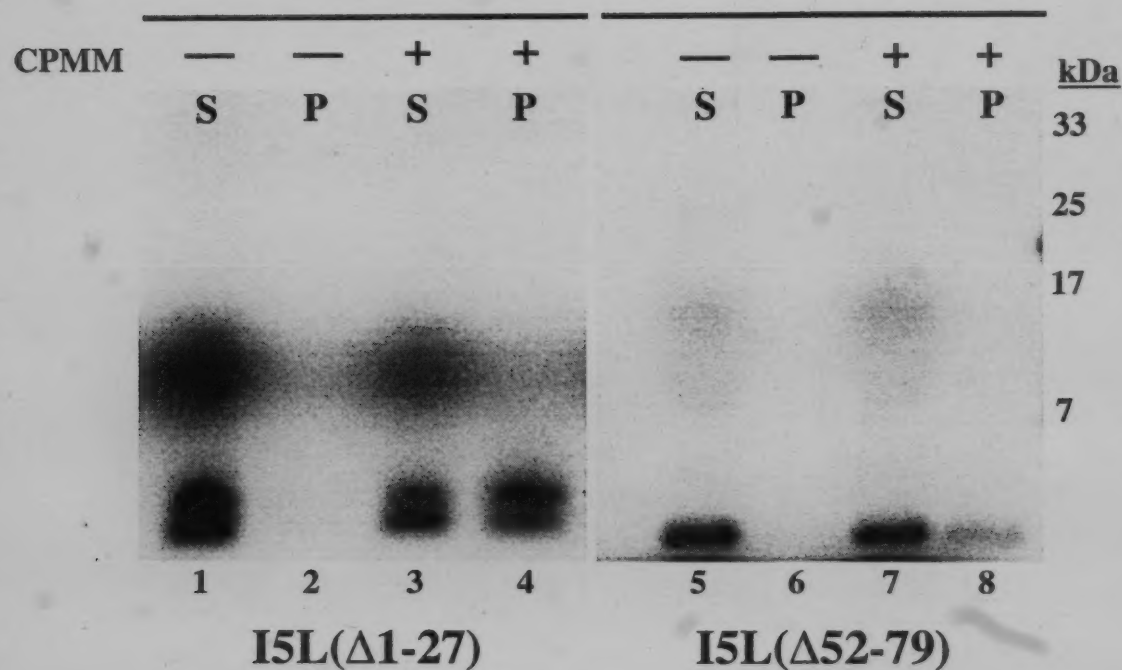


Figure 23. An N-terminal truncation of I5L inserts post-translationally, but a C-terminal truncation does not. A [^{35} S]-labelled N-terminal I5L truncation, I5L(Δ 1-27) (lanes 1-4), and a C-terminal I5L truncation, I5L(Δ 52-79) (lanes 5-8) were generated using a coupled *in vitro* transcription/translation assay. Canine pancreatic microsomal membranes (CPMM) were either not added (lanes 1, 2, 5 and 6), or added after addition of cycloheximide (lanes 3, 4, 7 and 8) in order to facilitate post-translational insertion. Microsomal membranes were separated from supernatants by centrifugation and both supernatant (S) and pellet (P) fractions were analyzed by SDS-PAGE.

insertion (refer to Figure 22, lanes 1-6) (Kim *et al.*, 1997). The doublet seen for this construct is likely the result of translation initiation from an alternative start codon. A candidate is methionine 42, which would express a fragment with an intact tail-anchoring motif.

The second truncation, I5L(Δ 52-79), construct had the C-terminal 28 amino acids deleted. This construct was effectively missing its C-terminal hydrophobic tail. Since deletion of the N-terminus did not prevent membrane association, we predicted that membrane integration would be abolished upon deletion of the C-terminus. In both the co- and post-translational assays, minimal membrane association of this construct was observed (Figure 23, lanes 5-8). There was a small proportion that associated with membrane post-translationally (lane 8), but this amount appeared to comprise only a small fraction of the total. From these results we concluded that the C-terminus was critical for membrane insertion *in vitro*, consistent with confocal results from uninfected cells in which Golgi localization was abolished when this hydrophobic tail was removed.

3.9 I5L and B6R are integral, not peripheral, membrane proteins

Separation of the soluble and membrane-associated protein fractions in our TNT assays was achieved by centrifuging the reactions in order to pellet the membranous material. Proteins associating with the microsomes could be anchored in the membrane as integral membrane proteins or loosely bound as a result of peripheral or non-specific membrane binding. In order to determine if I5L was actually anchored in the membrane, we made use of the common technique of washing the membrane pellet with an alkaline solution to disrupt membrane integrity (da Fonseca *et al.*, 2000). Upon alkaline washing, integral membrane proteins should remain in the pellet fraction, while loosely bound proteins should be released into the supernatant fraction. When co-translationally inserted full-length I5L was treated with an alkaline wash, no significant reduction of

radiolabelled I5L was seen between the treated and untreated pellet fractions (Figure 24, compare lanes 2 and 4). This suggests that the bulk of I5L found in the pellet is anchored in the microsomes.

When alkaline washing was performed on the N- or C-terminally truncated I5L constructs, again no significant amount of radiolabelled protein was removed from the pellet fraction (Figure 24, compare lanes 6 and 8, and 10 and 12, respectively). This suggests that these two constructs were also anchored in the membrane. It should be noted that although the C-terminally truncated construct showed evidence of membrane anchoring, very little of this translated product (<10%) actually found its way into the pellet fraction. This suggests that the hydrophobic domain does not play a major role in membrane anchoring of full-length I5L *in vitro*. The tail-anchored protein, B6R, also did not show appreciable washing from membranes, consistent with our classification of B6R as an integral, tail-anchored membrane protein (Figure 24, compare lanes 14 and 16).

3.10 I5L and B6R are membrane proteins, not aggregates

In order to determine that our constructs were actually anchored in the membranes, we performed another important control experiment. It was possible, especially considering the unusually high hydrophobicity of I5L, that our translated protein was actually aggregating in the supernatant instead of anchoring itself into membranes. Protein aggregates are dense, and so would be expected to pellet with the microsomes. It would also likely be resistant to alkaline washing and would thus behave just like an anchored protein in our assays. We needed an assay that could distinguish between membrane-associated proteins and aggregated proteins. For this we chose vesicle flotation analysis (Millman and Andrews, 2001). The principle behind vesicle flotation is that membrane-associated proteins can be separated from aggregated proteins on the basis of their relative densities. When a completed TNT reaction is placed at the

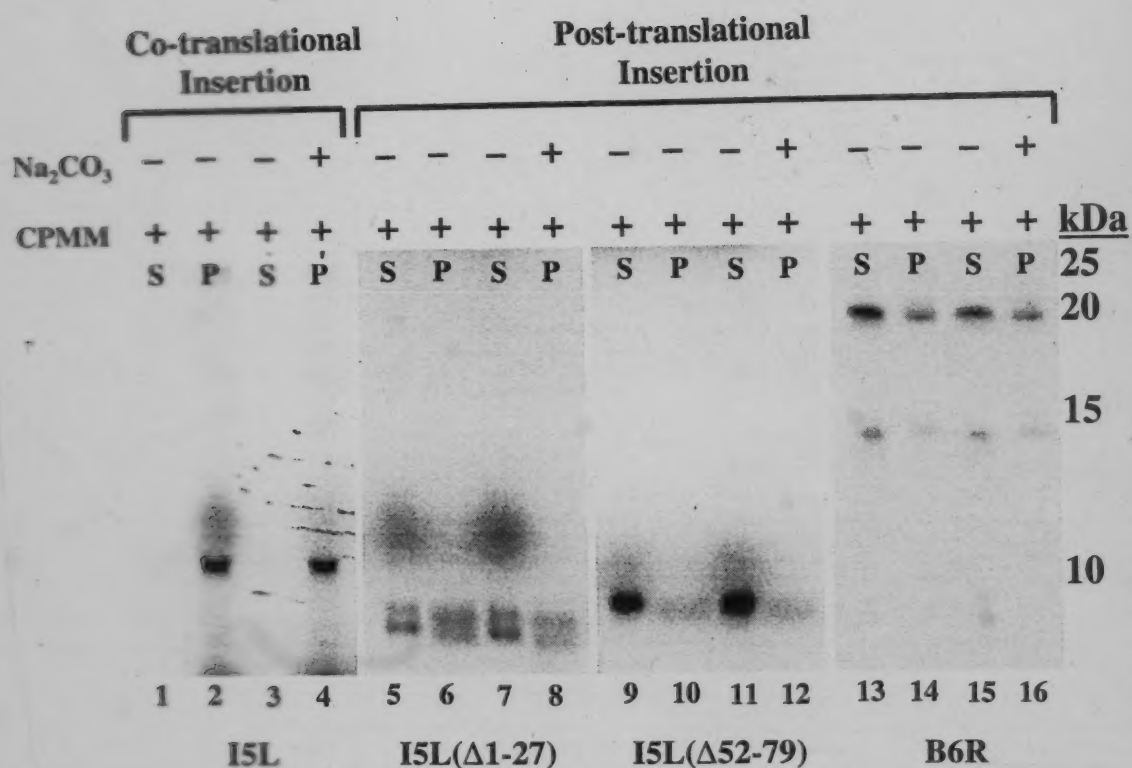


Figure 24. I5L and B6R are anchored into microsomal membranes

[³⁵S]-labelled proteins were generated using a coupled *in vitro* transcription/translation assay. Once co- or post-translational insertion was achieved by the addition of canine pancreatic microsomes (CPMM), samples were centrifuged to separate the supernatant (S) and pellet (P) fractions. Pellets were then washed with either PBS (-) or 0.1 M Na₂CO₃, pH 11.5 (+), recentrifuged, and analyzed by SDS-PAGE and autoradiography.

bottom of a dense sucrose gradient and centrifuged at high speeds, the membranous material, being less dense than aggregates, will float to the top of the gradient while denser aggregates will form a pellet at the bottom of the gradient. When co-translationally inserted I5L was centrifuged in such a gradient, it floated into the upper fractions where membrane proteins would be found (Figure 25, lanes 4 and 5). There was no appreciable amount of radiolabelled protein found in the pellet fraction where aggregates would be found (lane 7). When B6R was subjected to vesicle flotation analysis, it floated upwards with the membrane fraction, in accordance with its classification as a tail-anchored protein (Figure 26, lanes 4 and 5).

3.11 I5L localizes to the Golgi compartment in the absence of infection

Our data thus far had shown that although the N-terminus of I5L mediated factory localization during infection, it did not show an appreciable affinity for membranes in our *in vitro* translation assays. The C-terminus on the other hand was able to mediate Golgi compartment localization during infection if the N-terminus was missing. Furthermore, the C-terminus showed a high affinity for membranes *in vitro*, and was thus capable of anchoring I5L into membranes, even if the N-terminus was deleted. This raised the question of how the N-terminus is able to reach the viral factory if it does not display an ability to anchor into membranes. The simplest explanation was that the N-terminus requires an interaction with another viral protein in order to reach the factory, as is the case for several other virion components (Dyster and Niles, 1991; Vazquez *et al.*, 1998; Wolffe *et al.*, 2001). In order to test this hypothesis, we decided to investigate the subcellular targeting of I5L when it is expressed in isolation in uninfected cells. Since no other viral proteins would be present, we would be able to determine which features of I5L targeting are dependent upon other viral proteins and which are not.

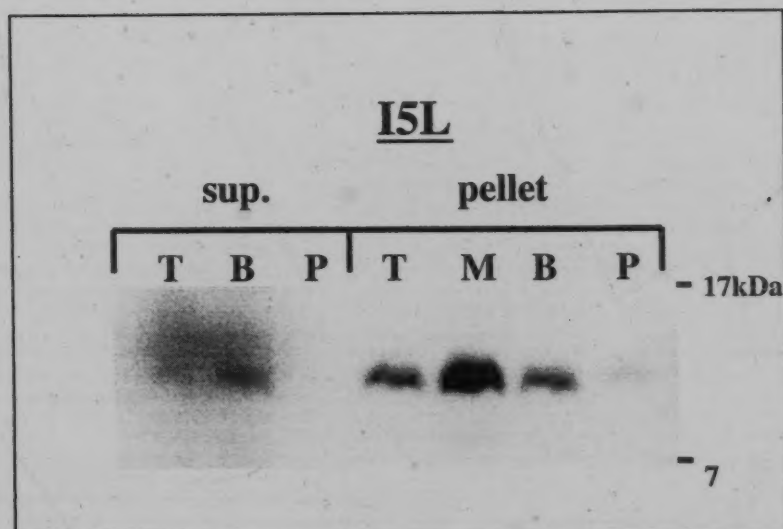


Figure 25. I5L is a membrane protein

[³⁵S]-labelled full-length I5L was generated using a coupled *in vitro* transcription/translation assay. Once co-translational insertion into microsomes was achieved, samples were centrifuged at low speed to separate the supernatant fraction from the pellet fraction. Supernatants and resuspended pellet fractions were then mixed with a sucrose solution to yield a 1.6M sucrose mixture. A sucrose gradient was completed by layering 1.25M and 0.25M layers onto the 1.6M layer which contained the radiolabelled sample. The supernatant and pellet fractions were run on separate gradients (named "sup." and "pellet" above). Samples were then centrifuged at high speed to allow for separation of the sample on the gradient based on the densities of the various components. The "sup." gradient was separated into three fractions, named top (T), bottom (B) and pellet (P). The P fraction was obtained by washing out the bottom of the gradient tube with SDS sample buffer. The pellet gradient was separated into four fractions, named top (T), middle (M), bottom (B) and pellet (P). Samples were then separated by SDS-PAGE and visualized by autoradiography.

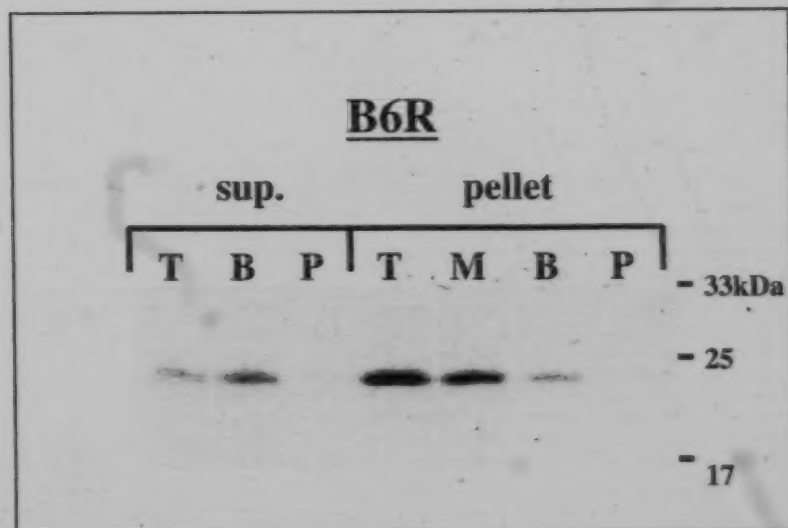


Figure 26. B6R is a membrane protein

[³⁵S]-labelled B6R was generated using a coupled *in vitro* transcription/translation assay. Once post-translational insertion into microsomes was achieved upon addition of canine pancreatic microsomes, samples were centrifuged at low speed to separate the supernatant fraction from the pellet fraction. Supernatants and resuspended pellet fractions were then mixed with a sucrose solution to yield a 1.6M sucrose mixture. A sucrose gradient was completed by layering 1.25M and 0.25M layers onto the 1.6M layer which contained the radiolabelled sample. The supernatant and pellet fractions were run on separate gradients (named "sup." and "pellet" above). Samples were then centrifuged at high speed to allow for separation of the sample on the gradient based on the densities of the various components. The "sup." gradient was separated into three fractions, named top (T), bottom (B) and pellet (P). The P fraction was obtained by washing out the bottom of the gradient tube with SDS sample buffer. The pellet gradient was separated into four fractions, named top (T), middle (M), bottom (B) and pellet (P). Samples were then separated by SDS-PAGE and visualized by autoradiography.

In order to study I5L localization in uninfected cells we once again chose to tag our proteins of interest with GFP and transfect them into uninfected cells. Unlike our studies in infected cells, where expression of our GFP fusions was controlled by a poxviral promoter, we placed this set of GFP fusions under the control of a ubiquitous CMV promoter and determined protein localization by confocal microscopy.

As a starting point we constructed a full-length I5L-GFP fusion. The decision of which terminus to which GFP should be fused was complicated by the presence of potential targeting motifs at both ends. At the N-terminus was the putative signal sequence and at the C-terminus was the putative tail-anchoring motif. Fusion of GFP to either end of I5L may have unpredictable consequences and may inhibit the targeting we wish to observe. Therefore we decided to generate both constructs in which GFP was fused to either the N-terminus or the C-terminus.

When HeLa cells were transiently transfected with a GFP control plasmid and analyzed by confocal microscopy, a diffuse staining pattern characteristic of small, soluble proteins was observed (Figure 27, panel B). Conversely I5L-GFP, in which GFP was fused to the C-terminus, was found to localize to a perinuclear region in live, transfected cells (Figure 27, panel E) which partially overlapped with the endoplasmic reticulum fluorophore, ER Tracker (Figure 27, panels D and F). Although I5L-GFP colocalized with ER Tracker, it was concentrated in a perinuclear region which is more characteristic of Golgi compartment localization (Figure 27, panel F). To further characterize the observed I5L-GFP localization, transfections were repeated using a fluorescent cationic lipid dye, GolgiTracker, which labels all subcellular membranes to some extent, but preferentially accumulates in the Golgi compartment (manufacturer's product sheet). Upon transfection of HeLa cells with I5L-GFP followed by subsequent staining with GolgiTracker, I5L-GFP displayed virtually perfect colocalization with the Golgi compartment (Figure 28, panels D-F), while cells transfected with a GFP control

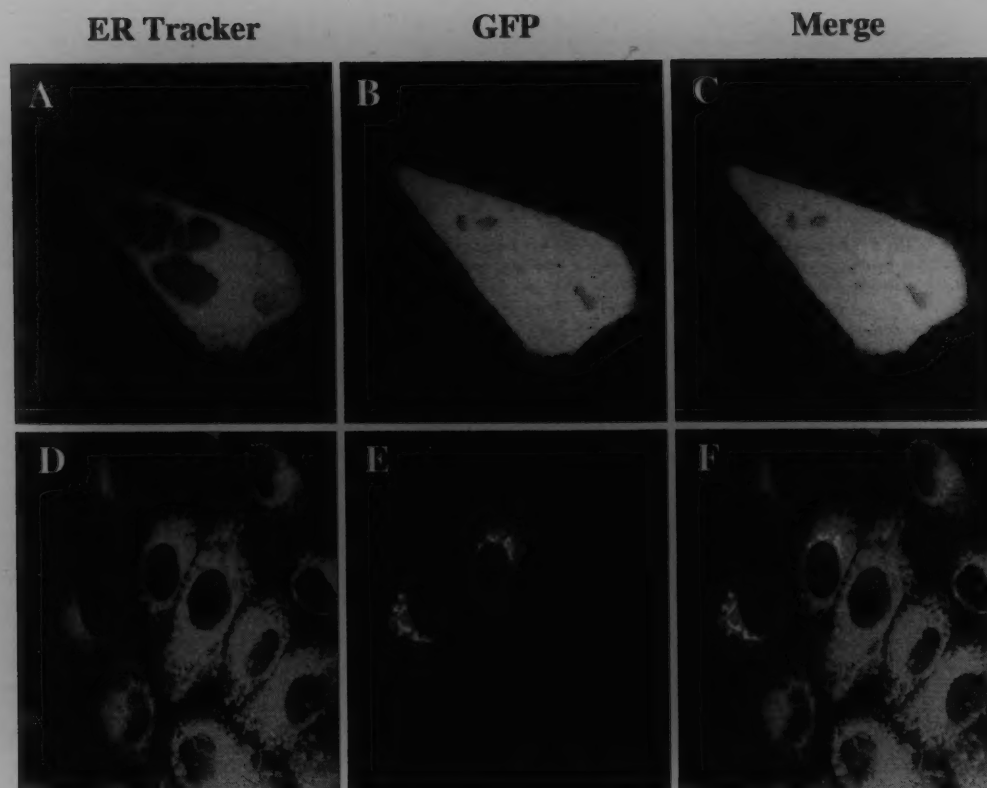


Figure 27. Full length I5L with GFP fused to the C-terminus localizes to a perinuclear region in uninfected cells. HeLa cells were transiently transfected with either GFP (panels A-C), or a construct expressing I5L with a C-terminal GFP tag (panels D-F). Live cells were stained with ER Tracker and analyzed by confocal microscopy. Two separate images were obtained for each cell: the ER Tracker emission (panels A and D), and GFP emission (panels B and E). A merged image of ER Tracker and GFP are provided (panels C and F).

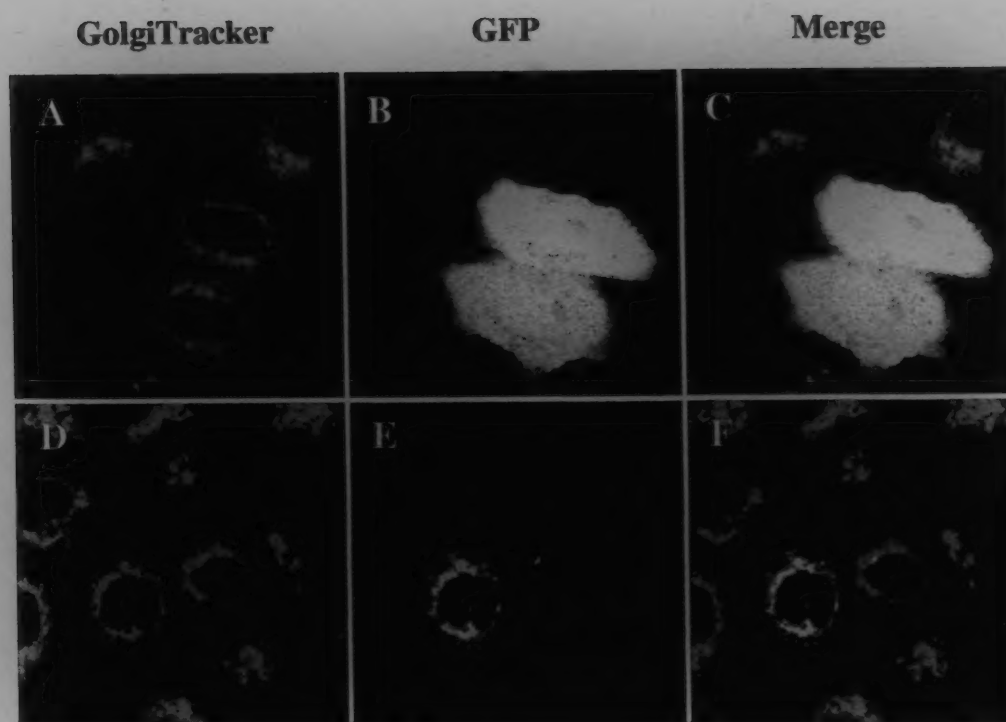


Figure 28. I5L with GFP fused to the C-terminus localizes to the Golgi compartment in uninfected cells. HeLa cells were transiently transfected with either GFP (panels A-C), or a construct expressing I5L with a C-terminal GFP tag (panels D-F). Live cells were stained with GolgiTracker and analyzed by confocal microscopy. Two separate images were obtained for each cell: the GolgiTracker emission (panels A and D), and GFP emission (panels B and E). A merged image of GolgiTracker and GFP are provided (panels C and F).

plasmid displayed a diffuse staining pattern (Figure 28, panels A-C). We concluded from these observations that I5L was a Golgi-localized protein in uninfected cells, reminiscent of Golgi-compartment localization of N-terminally-truncated I5L in infected cells.

We wanted to verify Golgi localization in another cell-line. For this purpose we chose baby hamster kidney (BHK) cells which contain large, prominent Golgi complexes (Dr. T. Hobman, personal communication). BHK cells were transiently transfected with I5L-GFP, and Golgi complexes were visualized by fixing and staining with a polyclonal mannosidase II antibody which is specific for the *cis*-medial Golgi compartment (Novikoff *et al.*, 1983; Futerman *et al.*, 1990) (Figure 29). As with live HeLa cells, fixed BHK cells displayed a diffuse, cell-wide GFP staining pattern when transfected with a GFP control plasmid (Figure 29, panels A-D). When transfected with I5L-GFP, BHK cells displayed highly specific Golgi targeting as assessed by colocalization with the mannosidase II antibody (Figure 29, panels E-H), corroborating our previous observations in live HeLa cells.

In order to assess the effect that an N-terminal GFP tag would have on Golgi localization, we fused GFP to the N-terminus of I5L creating GFP-I5L. BHK cells were transfected with this construct and fixed prior to staining with a mannosidase II polyclonal antibody (Figure 30). When this construct was analyzed, Golgi localization was surprisingly still evident (Figure 30, panels A-C), although non-Golgi staining was also evident. Although most of the GFP-I5L was localized to the Golgi compartment, the remainder was suggestive of ER localization (Figure 30, panels D-F). The relative amounts of Golgi and ER-like targeting were variable from cell to cell, ranging from almost exclusive Golgi localization (Figure 30, panels A-C) to almost exclusive ER-like localization (Figure 30, panels D-F). Whether the small amount of ER-like targeting was the result of ER-to-Golgi trafficking or whether it was due to mistargeting of an overexpressed GFP construct is unclear. Nonetheless, the fact that the N-terminal GFP

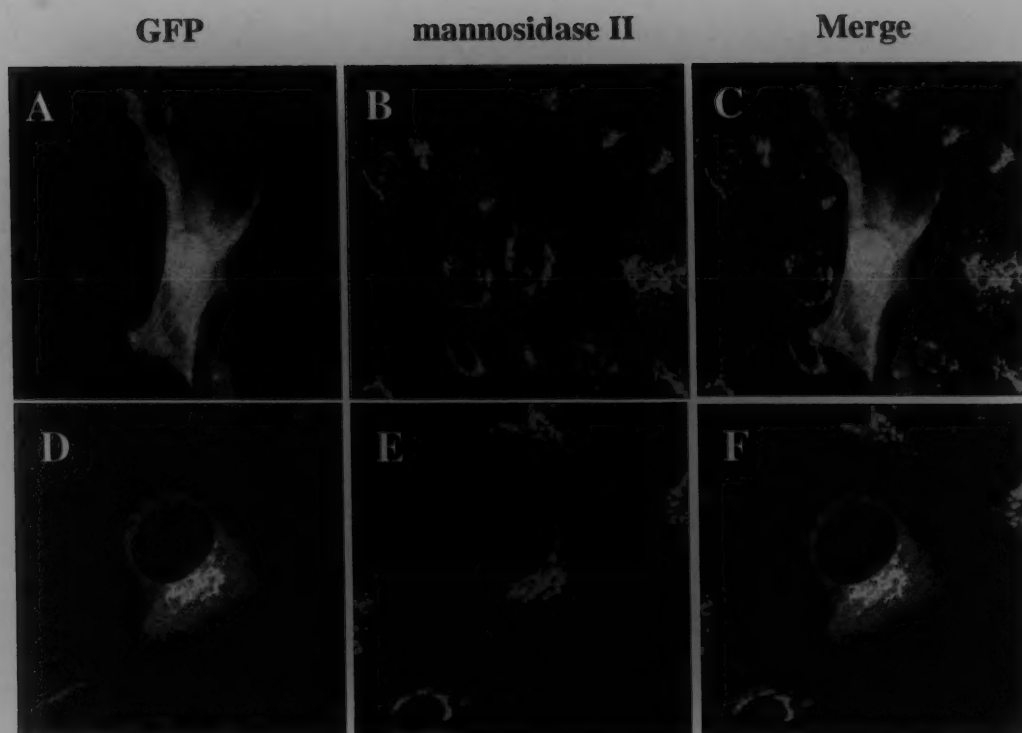


Figure 29. I5L with GFP fused to the C-terminus localizes to the Golgi compartment in uninfected BHK cells

Confocal microscopy of BHK cells transiently transfected with either GFP alone (panels A-C) or an I5L-GFP fusion (panels D-F) in the absence of infection. Following transfection, cells were fixed and stained with a polyclonal mannosidase II antibody which is specific for the Golgi compartment. The secondary antibody was an Alexa-conjugated goat anti-rabbit IgG antibody. Two separate images were obtained for each cell GFP emission (panels A and D) and Alexa emission (panels B and E). A merged image of GFP and Alexa emissions are provided (panels C and F).

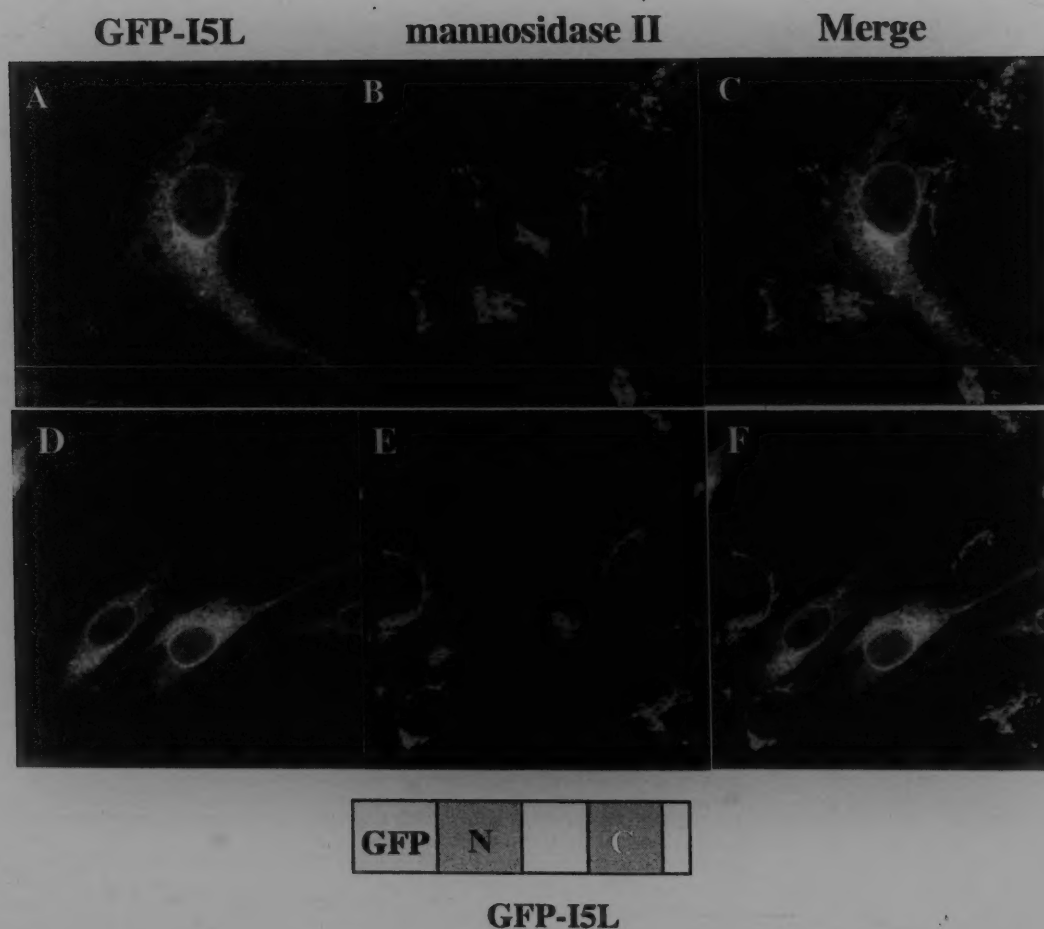


Figure 30. I5L with GFP fused to the N-terminus targets to the Golgi with less efficiency than the C-terminal fusion.

Confocal microscopy of BHK cells transiently transfected with a full-length GFP-I5L fusion (panels A-F). GFP is fused to the N-terminus of I5L. Two representative cells are shown in panels A-C and D-F, respectively. The cells in both sets of panels were transfected in parallel with the identical construct in an identical manner. Following transfection, cells were fixed and stained with a polyclonal mannosidase II antibody which is specific for the Golgi compartment. The secondary antibody was an Alexa-conjugated goat anti-rabbit IgG antibody. Two separate images were obtained for each cell: GFP emission (panels A and C) and Alexa emission (panels B and E). A merged image of GFP and Alexa emissions are provided (panels C and F).

tag interfered with Golgi targeting suggested that the hydrophobic N-terminus may be a targeting motif.

3.12 The C-terminal hydrophobic domain is necessary for Golgi targeting, while the N-terminal hydrophobic domain is not

In order to assess the relative contribution(s) of the N- and C-termini to Golgi targeting in uninfected cells, a series of I5L truncations were fused to GFP, transiently expressed in HeLa or BHK cells, and analyzed by confocal microscopy. The two most revealing truncations, I5L(Δ 1-27)GFP and I5L(Δ 52-79)GFP had the putative signal sequence or C-terminal tail deleted, respectively.

When HeLa cells were transfected with an N-terminal truncation I5L(Δ 1-27)GFP and stained using GolgiTracker, the 'signalless' construct localized to the Golgi compartment very effectively (Figure 31, panels A-C). Little or no GFP was detected in other membranes indicating that targeting to the Golgi was very efficient. These results suggested that the putative signal sequence was not necessary for Golgi targeting. Since deletion of the N-terminus did not noticeably affect Golgi localization, deletion of the putative C-terminal tail-anchoring domain was expected to have an impact on targeting. HeLa cells were transfected with the C-terminal truncation, I5L(Δ 52-79)GFP and stained with Golgi Tracker (Figure 31, panels D-F). Deletion of this domain completely abolished membrane localization and resulted in a GFP distribution indistinguishable from soluble GFP.

When I5L(Δ 1-27)GFP and I5L(Δ 52-79)GFP were examined in fixed BHK cells, I5L(Δ 1-27) retained its ability to localize to the Golgi compartment (Figure 32, panels A-C), while I5L(Δ 52-79) did not (Figure 32, panels D-F). This corroborated what was seen with live HeLa cells. The observation that the C-terminus of I5L is necessary for membrane targeting is characteristic of other vaccinia tail-anchored proteins such as B6R

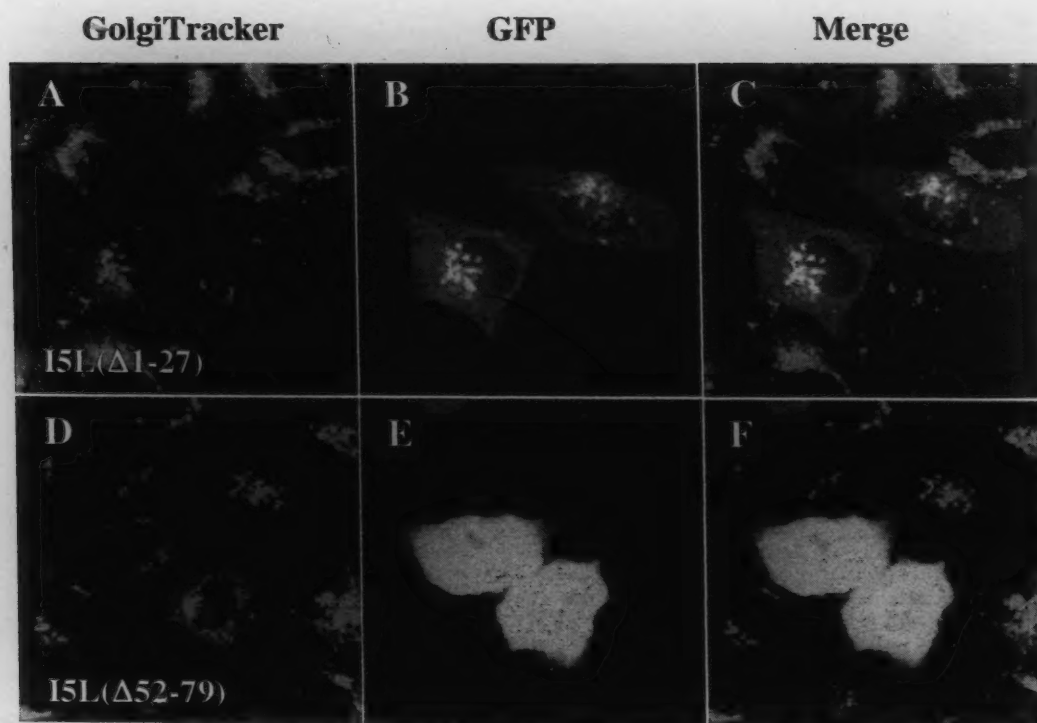


Figure 31. The hydrophobic C-terminus of I5L is necessary for Golgi localization in uninfected cells, while the hydrophobic N-terminus is not. HeLa cells were transiently transfected with either an N-terminally truncated I5L, I5L(Δ 1-27)GFP (panels A-C) or a C-terminally truncated I5L, I5L(Δ 52-79)GFP (panels D-F). Both truncations possessed a GFP tag at the C-terminus. Live cells were stained with GolgiTracker and analyzed by confocal microscopy. Two separate images were obtained for each cell: the GolgiTracker emission (panels A and D), and GFP emission (panels B and E). A merged image of GolgiTracker and GFP are provided (panels C and F).

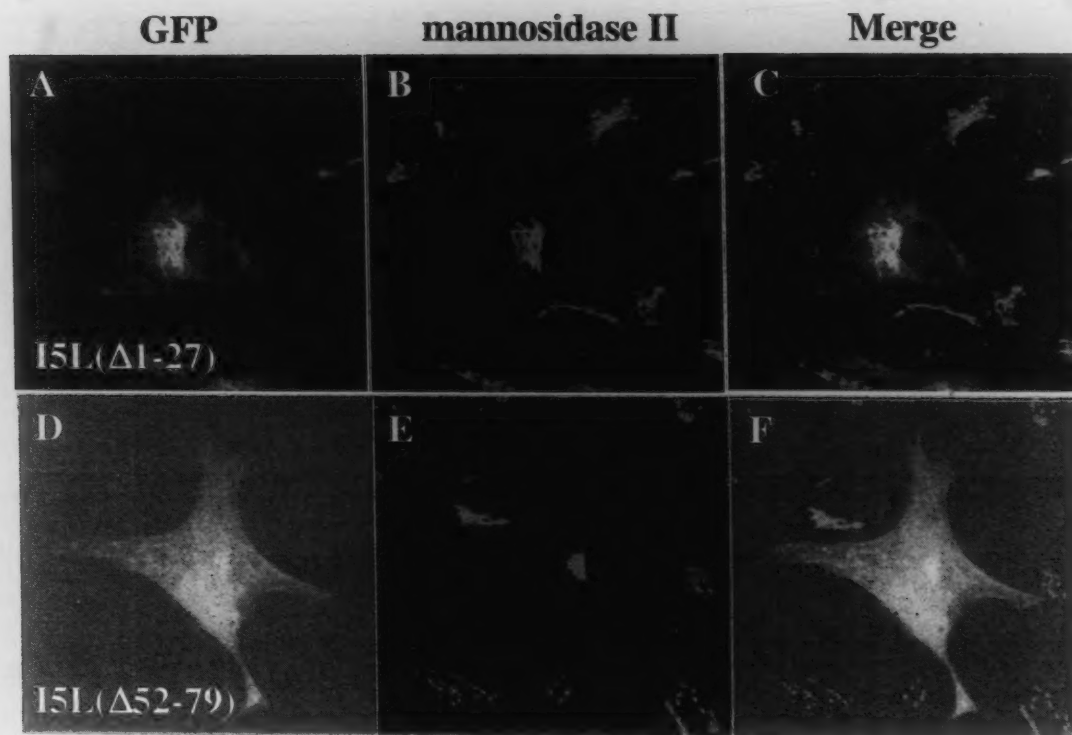


Figure 32. The C-terminal tail of I5L is critical for Golgi localization in uninfected BHK cells while the N-terminus is not.

Confocal microscopy of BHK cells transiently transfected with either an N- or C-terminally truncated I5L-GFP fusion. The N-terminal I5L truncation (panels A-C) expresses residues 28-79, while the C-terminal truncation (panels D-F) expresses residues 1-51. In both constructs, GFP was fused to the C-terminus. Following transfection, cells were fixed and stained with an α -mannosidase II polyclonal antibody which is specific for the Golgi compartment. The secondary antibody was an Alexa-conjugated goat anti-rabbit IgG antibody. Two separate images were obtained for each cell: GFP emission (panels A and D) and 2° antibody emission (panels B and E). A merged image of GFP and 2° antibody emissions are provided (panels C and F).

(S. Wasilenko and M. Barry, unpublished data), F1L (T. Stewart and M. Barry, unpublished data) and H3L (da Fonseca *et al.*, 2000), in that deletion of the C-terminal targeting domain abolishes membrane localization.

3.13 The 7-amino acid tail of I5L is not necessary for Golgi targeting

To further characterize the targeting role of the regions adjacent to the C-terminal hydrophobic domain, a GFP fusion was made in which the 7-amino acid positively charged tail downstream of the C-terminal hydrophobic domain was deleted, yielding construct I5L(Δ 70-79)GFP. These short tails, which often contain positive charges, have been shown to be important for some tail-anchored proteins to reach their target membranes efficiently (Wattenberg and Lithgow, 2002; Horie *et al.*, 2002). This is especially true of those destined for the mitochondria (Pedrazzini *et al.*, 2000). When BHK cells were transfected with this construct and fixed, specific Golgi localization was still observed as evidenced by its perfect overlap with the mannosidase II antibody (Figure 33, panels A-E). This result indicated that this short polar tail was not needed for Golgi targeting of I5L.

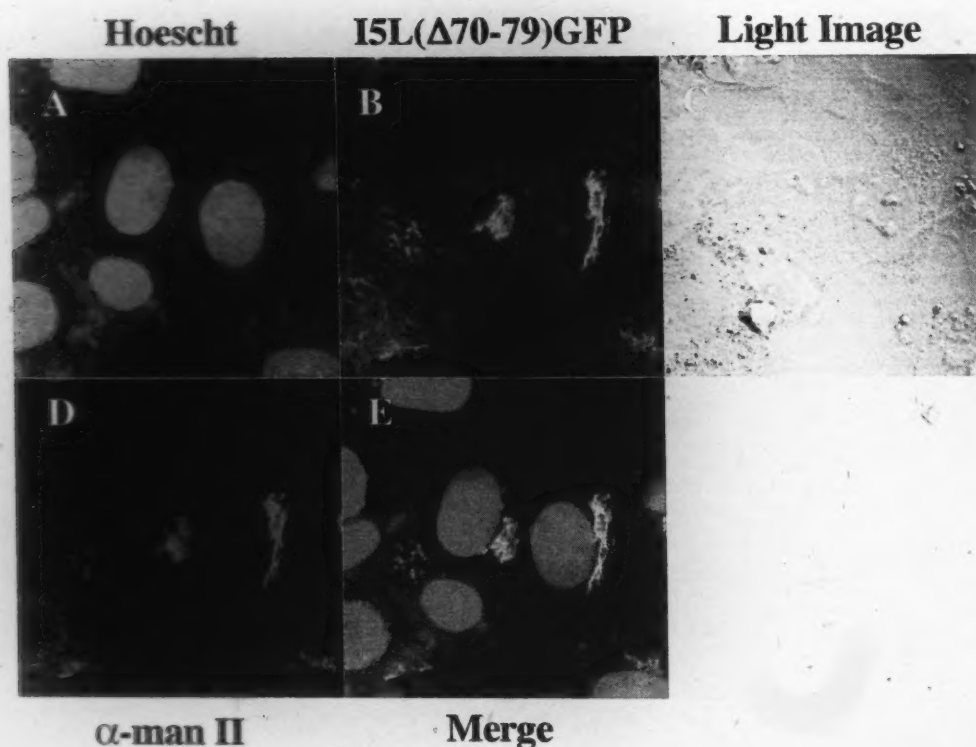


Figure 33. The short C-terminal tail of I5L is not essential for Golgi localization

Confocal microscopy of BHK cells transiently transfected with either a C-terminally truncated I5L-GFP fusion. The C-terminal truncation (panels A-D) is missing residues 70-79 and has GFP fused to the C-terminus. Following transfection, cells were fixed with and stained with an α -mannosidase II polyclonal antibody which is specific for the Golgi compartment. The secondary antibody was an Alexa-conjugated goat anti-rabbit IgG. Several different emissions were visualized: Hoescht 33342 (panel A), GFP emission (panel B), and light image (panel C), and a mannosidase II polyclonal antibody (panel D). A merged image is also shown (panel E).

4. Discussion

At the outset of this study, a question of great interest to us was the importance of I5L to vaccinia virus during infection. A common approach to answering such questions is to construct a genetic knockout and characterize the resulting phenotype. The amenability of poxviruses to genetic manipulation makes construction of knockouts a practical option for studying gene function. The most common technique, and the one employed by our laboratory, involves disruption of the target gene by a cassette which expresses a selectable marker. This cassette is engineered, using standard molecular biological techniques, to contain 5' and 3' fragments of the target gene at each end. The resulting cassette is then transfected into an appropriate mammalian cell line, after which transfected cells are infected by wild-type virus. This allows for homologous recombination of the cassette into the target gene of wild-type virions during infection. Although such recombination is probably not very efficient, the selection marker in the cassette allows for amplification of desired recombinants amidst a background of wild-type virus. The selection marker we used was the *E. coli* guanine phosphoribosyltransferase (*gpt*) gene (Falkner and Moss, 1988; Falkner and Moss, 1990; Isaacs *et al.*, 1990). Mycophenolic acid, an inhibitor of purine metabolism, is able to block the replication of vaccinia virus in normal cell lines. Recombinant viruses expressing *gpt* are able to grow in the presence of mycophenolic acid, and can thus form large, easily isolated plaques in tissue culture.

This general knockout procedure has been used successfully in our laboratory for several other poxviral proteins. After three separate unsuccessful attempts to isolate a recombinant I5L knockout, we have come to the conclusion that construction of an I5L knockout by this method may not be possible. The most obvious explanation is the possibility that I5L is essential for some stage of the virus lifecycle. Simply knocking out the gene with the *gpt* cassette may yield a non-viable virus -- a situation analogous to a

lethal knockout in eukaryotic systems. Other methods may have to be employed to determine the function of I5L during infection, such as the construction of an IPTG-inducible knockout.

One of the initial hypotheses of this study was that I5L is a viral protein important for virion morphogenesis. The basis for this hypothesis includes (1) the reported presence of I5L in the virion particle (Takahashi *et al.*, 1994), (2) the presence of I5L in the central region of the poxviral genome which is rich in 'housekeeping' genes, (3) the presence and high sequence conservation of I5L in all vertebrate poxviruses, (4) the lack of an obvious catalytic motif, (5) the fact that I5L is under the control of a late promoter, a common feature of virion structural proteins, and (6) the high hydrophobicity of I5L, another common feature of virion structural proteins. All of the data accumulated in this study are consistent with our initial hypothesis. This includes the successful cloning and sequencing of an I5L orthologue from ectromelia virus, which is consistent with its classification as an essential poxviral gene. In addition, our inability to construct an I5L knockout is consistent with the idea that this protein is essential. We have also demonstrated that I5L is found in viral factories, which is a prerequisite for its incorporation into the IMV particle, and that I5L is physically capable of anchoring into membranes. All of these observations are consistent with the hypothesis that I5L is a structural IMV protein.

Our results demonstrate that during infection, I5L associates with viral factories in a manner dependent upon the N-terminal hydrophobic domain. The hydrophobicity of this domain suggests that it is membrane-associated, but using TNT assays we were only able to demonstrate membrane association of I5L when the C-terminal hydrophobic domain was present. The fact that a GFP fusion lacking the C-terminal membrane 'anchor' was still able to efficiently localize to viral factories suggests that the N-terminus is somehow able to mediate factory localization on its own. Either the N-

terminus has a high affinity for viral membranes during infection, and this cannot be recapitulated using microsomes *in vitro*, or the N-terminus localizes to viral factories via protein-protein interactions with another viral protein, or proteins. There is a precedent for such protein-protein interactions during vaccinia infection. The IMV membrane component, A27L, does not interact directly with IMV membranes, but instead binds directly with the integral IMV membrane protein A17L (Vasquez *et al.*, 1998). In the case of D2L and D3R IMV structural proteins, both D2L and D3R are only able to enter nascent virion cores if the other is also expressed (Dyster and Niles, 1991). Temperature-sensitive lesions in either D2L or D3R prevent both from inserting into nascent cores (Dyster and Niles, 1991). In a third example, the EEV membrane protein, A33R, is needed to target A36R to the EEV via direct protein-protein interactions. If A33R is not expressed, A36R targets instead to the Golgi compartment (Wolffe *et al.*, 2001). It is therefore possible that the N-terminus of I5L localizes to viral factories via interactions with other viral proteins and not via integration into viral membranes.

From our data, it is not clear what the role of the C-terminus of I5L might be during infection. While it is clearly not needed to target I5L to the factory during infection, the extremely high conservation of this domain argues that it is important. The C-terminus was sufficient for efficient localization of GFP to the Golgi compartment in uninfected cells, and TNT assays confirmed that this domain is anchored into membranes. It therefore seems likely that this domain is also interacting with membranes during infection, but the identity of such membranes is unknown.

It is interesting that in uninfected cells, I5L localizes to the Golgi compartment. Although such localization may be an artifact of expressing I5L in the absence of other viral proteins, one cannot ignore the fact that membranes of the ERGIC and/or Golgi compartment are the likely source for IMV membrane precursors. Perhaps I5L initially localizes to the ER, ERGIC or Golgi compartment during infection and is consequently

shipped to the viral factory in a process mediated by the N-terminus (Figure 34). Such a model would predict that deletion of the N-terminus would result in a C-terminal fragment that is trapped at some stage in the ER-Golgi secretory pathway. This is in fact exactly what we observed when the N-terminus was deleted -- the C-terminal fragment remained in the Golgi compartment. This situation is reminiscent of A33R and A36R, in that prevention of an interaction between these two proteins causes A36R to target to the Golgi compartment instead of the viral membranes (Ward *et al.*, 2003).

Since I5L appears to have two 'business ends' it may serve as a linker between two virion components during infection. The C-terminus is likely interacting with a membrane, while the N-terminus may be interacting with a membrane or another viral protein. Since targeting of I5L to the Golgi compartment in uninfected cells is not altered upon subsequent infection with wildtype virus, such an interaction would likely occur prior to arrival of I5L in the Golgi compartment. It is known that wrapping of viroplasmic material by crescent membranes requires a direct interaction between crescent membranes and viroplasm, but the protein(s) responsible for this process are poorly defined. One protein involved in this stage of virion assembly is A10L (Heljasvaara *et al.*, 2001). Repression of A10L expression results in the accumulation of spherical immature virions partially filled or completely devoid of viroplasm. The absence of A10L expression somehow prevents newly forming crescent membranes from wrapping viroplasm. A similar phenotype was found using A30L inducible knockouts (Szajner *et al.*, 2001a). Repression of A17L expression prevents recruitment of viral membranes to the factory (Rodriguez *et al.*, 1995), and repression of the A17L binding partner, A14L, prevents recruited membranes from interacting with viroplasm (Rodriguez *et al.*, 1997; Rodriguez *et al.*, 1998). An inducible knockout of I5L may yield a similar phenotype to these proteins, and is currently being constructed.

Proposed Model of I5L Localization

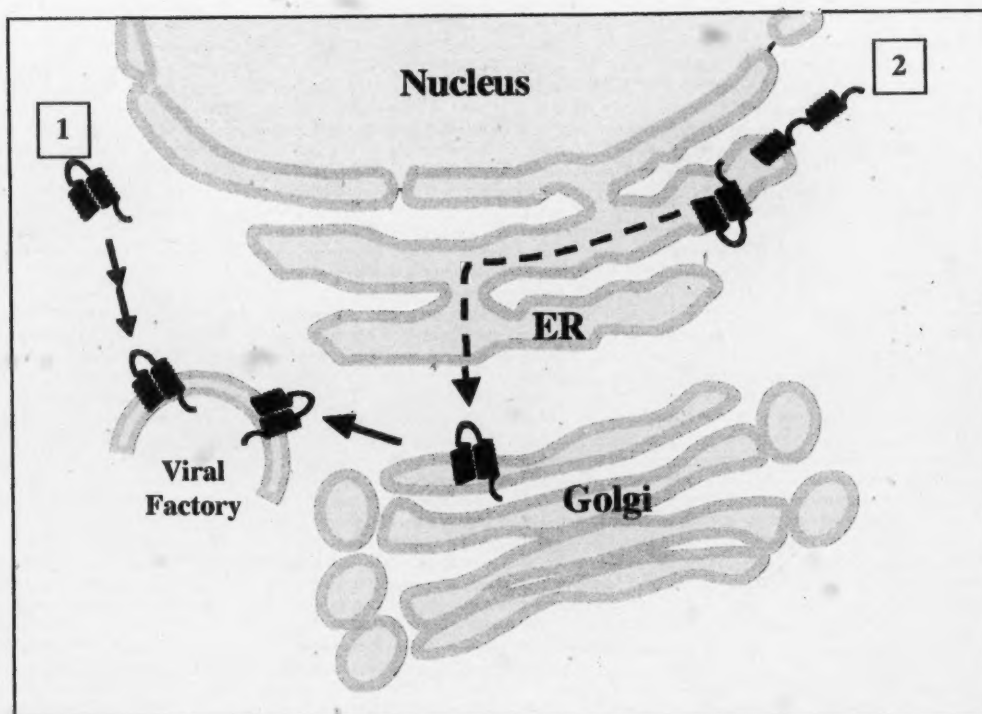


Figure 34. Proposed Model of I5L Localization

Shown above are two potential routes indicating how I5L might reach viral factories during infection. In mechanism 1, I5L targets directly to viral factories from the cytoplasm in a process mediated by the N-terminus. The inability of I5L to interact with membranes *in vitro* suggests that association of I5L with viral factories is dependent upon interaction of the N-terminus with another viral protein. The resulting orientation of I5L is unclear. In the second route, I5L is first inserted into the endoplasmic reticulum where it traverses the ER-to-Golgi membrane system prior to its eventual incorporation into crescent membranes in viral factories. By this route, anchoring into membranes is mediated by the hydrophobic C-terminus, whereas association with the factory is mediated by the N-terminus.

One particularly interesting feature of I5L localization to viral factories is the distinct and mutually exclusive colocalization of I5L and G1L within the factory. I5L and G1L appeared to accumulate in distinct, but tightly packed clusters suggestive of organization of the factory into discrete subdomains. Such subdomains have been reported by several groups utilizing electron microscopy (Heljasvaara *et al.*, 2001; Szajner *et al.*, 2001; Sancho *et al.*, 2002). The factory is often described as having two main components: viroplasmic material rich in core proteins and viral genomic DNA, and crescent material rich in IMV membrane proteins and components of the core wall (Sarov and Joklik, 1973). Under electron microscopy, large masses of viroplasm within the factory become surrounded by newly forming crescents (Dales and Mosbach, 1968; Sancho *et al.*, 2002). The immediate implication is that I5L is localizing to crescent membrane regions since it displays a high affinity for membranes in our *in vitro* translation assays. G1L, being a core proteinase and lacking an obvious transmembrane region would be expected to target to the viroplasmic fraction. Generation of an I5L antibody would allow immunoelectron microscopy studies to definitively show if I5L and G1L target to the predicted subdomains of the viral factory.

When this study began, a central hypothesis was that I5L was a tail-anchored protein. This hypothesis was mainly fueled by the fact that the C-terminus of I5L resembles the tail-anchoring motifs of several confirmed tail-anchored proteins, including the myxoma M11L protein (Everett *et al.*, 2000), the BHRF-1 of Epstein-Barr virus (Khanim *et al.*, 1997), and Bcl-2 (Kim *et al.*, 1997) among others. The C-terminal motif of I5L also resembled anchoring motifs of several vaccinia tail-anchored proteins including B6R, F1L and H3L. It therefore seemed reasonable that I5L was a tail-anchored protein. However, upon analysis of our data, it has been difficult to classify I5L as such. In some aspects it behaves as one, and in other aspects it does not. In support of the classification of I5L as a tail-anchored protein is the observation that when I5L was

expressed in isolation, in uninfected cells, the C-terminal transmembrane domain was critical for membrane targeting, while the N-terminal transmembrane domain was not. This observation was consistent with vaccinia virus tail-anchored proteins B6R, F1L and H3L, for which membrane targeting was mediated solely by the C-terminal hydrophobic region. However, using *in vitro* translation assays, I5L was only able to insert into microsomes co-translationally which is inconsistent with a tail-anchored protein. Interestingly, post-translational insertion of I5L was seen if the N-terminus was deleted since TNT assays of this N-terminally truncated construct showed that approximately half of the radiolabelled protein was anchored in the microsomal membranes via a post-translational event, a pattern of insertion seen with a B6R control. Nonetheless, showing that the C-terminus, with the N-terminus deleted, can insert into microsomes post-translationally in an *in vitro* assay, and demonstrating that such insertion is relevant in a biological setting, particularly during infection, are two different things. To this point we have not shown if I5L inserts post-translationally into membranes during infection, a requirement for its classification as a tail-anchored protein.

Taken together, our results support a model in which I5L is expressed late during viral infection. I5L then localizes to the viral factory in a process mediated by the N-terminal portion of the protein (this model is discussed, as is an alternative targeting pathway in Figure 34). The C-terminal half of I5L likely interacts with membranes before recruitment to the factory, or interacts with viral membranes within the factory. I5L is also expressed at the right time and place to serve as a viral structural protein, consistent with previous reports that I5L is found in virion membranes. As well, the inability to construct an I5L knockout suggests that it is an essential viral protein.

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